EXPLORING THE BACTERIAL DIVERSITY OF THE MALE URETHRA DURING IDIOPATHIC URETHRITIS

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DEDICATION

To my mentor Dave Nelson and my friends in the Nelson Laboratory. It has been a privilege to learn from you and work with you over the past two years.

To my dear friend Arka. Thank you for getting me through the past two years with my sanity intact.

To my family. Thank you for all the love and support.

To my mother Erin. Thank you for everything.

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EXPLORING THE BACTERIAL DIVERSITY OF THE MALE URETHRA DURING IDIOPATHIC URETHRITIS

Idiopathic urethritis (IU) comprises up to 50% of symptomatic cases of male urethritis in clinical settings. The syndrome is of an unknown etiology but may be due to an as yet unidentified bacterial pathogen(s). We were interested in identifying pathogens that could cause IU using multiple methods. Shotgun metagenomic sequencing or 16S rRNA sequencing methods can provide rich datasets but are limited by the completeness of the corresponding sequence reference databases. We generated metagenomic and 16S datasets from DNA extracted from urethral swabs of men with IU to determine the composition of their urethral microbiome. In order to enrich the corresponding reference databases used to identify the reads in the sequence datasets, I cultivated bacteria from the first void urine (FVU) of men with IU. My goal was to grow and whole genome sequence bacterial isolates that are not currently represented in the reference databases.

Of the 216 men we enrolled at the Bell Flower STD clinic in Indianapolis, IN, 59 men had IU. I grew a total of 802 isolates from the FVU of the IU patients and identified those isolates using colony-based 16S rRNA PCR. Based on % sequence similarity to the nearest type strain, I sorted the 16S alleles into four categories: Species (≥98 % identity) (N=264), Genus (≥95 % identity) (N=407), Closest Match (<95 % identity) (N=95), and No Hit (0 % identity) (N=22). There were 24 genera represented in the isolate collection. Of these, the six most abundant genera were *Streptococcus, Staphylococcus, Corynebacterium, Haemophilus, Gardnerella,* and *Prevotella.* These six genera composed nearly 80% of all IU-associated isolates. All sequences below 98% sequence

similarity represent potentially novel strains of bacteria. We will proceed with whole genome sequencing of bacterial isolates with the goal of improving genome database coverage of bacterial diversity in the male urethra.

David E. Nelson, PhD, Chair

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LIST OF ABBREVIATIONS

CT – *Chlamydia trachomatis*

HSV – Herpes Simplex Virus

IU – Idiopathic Urethritis

MG – *Mycoplasma genitalium*

NAAT – Nucleic Acid Amplification Test

NG – *Neisseria gonorrhoeae*

NGU – Nongonococcal Urethritis

PMNLs – Polymorphonuclear Leukocytes

rRNA – Ribosomal Ribonucleic Acid

UU – *Ureaplasma urealyticum*

TV – *Trichomonas vaginalis*

Introduction

1.1 Male Urethritis

Male urethritis is an inflammatory syndrome of the male urethra. The urethra in men is the long, narrow tube that carries semen from the ejaculatory ducts and urine from the bladder through the length of the penis as they exit the body. The urethra can become inflamed when it is irritated or infected, causing the affected individual significant discomfort. Male urethritis is the most frequently diagnosed condition of the male urogenital tract and accounts for well over two million medical visits per year in the United States. [2] There are multiple infectious etiologies of male urethritis, the majority of which are sexually transmitted infections (STI) that can bear significant risk for sexual partners of urethritis patients. The risks to sexual partners of men with urethritis include severe complications of cervicitis or rectal infections and an increased risk of HIV acquisition. [11] In men, urethritis can evolve to the more serious conditions epididymitis and prostatitis. Epididymitis results in swelling and tenderness of the epididymis and nearby tissues including the involved testicle and inguinal lymph nodes. Prostatitis may cause difficulties with sexual dysfunction, urination, lower back pain, and lower abdominal or pelvic pain. [47]

All cases of urethritis are clinically diagnosed on the basis of observed inflammation and symptoms. The Centers for Disease Control and Prevention (CDC) suggests that clinicians consider microscopic evidence of two or more polymorphonuclear leukocytes (PMNLs) on a urethral gram stain (or ten PMNLs per high-powered field in sediment from spun first void urine) as sufficient for diagnosis. To prepare these slides, a health professional will insert a small swab about 1 cm into the

distal urethra and gently swirl to collect material. Observed mucoid or mucopurulent discharge from the penis on examination is also sufficient for a diagnosis of urethritis. Etiological information is typically obtained using nucleic acid amplification tests (NAAT) on first void urine (FVU) samples or by culture methods. Additional symptoms of male urethritis include dysuria, redness, swelling, and general irritation of the penis.

Male urethritis canonically divides into two categories based on etiology; gonococcal urethritis is caused by *Neisseria gonorrhoeae* and all other cases due to infectious etiologies are collectively referred to as non-gonococcal urethritis, or NGU. *N. gonorrhoeae* was established as a pathogen in urethritis and cervicitis by the late 1800s. As early as 1907, chlamydial particles were isolated from the cervices of women in investigations of Trachoma and the passage of the disease from mother to infant during childbirth. [55] By the 1960s, it was well documented that the bacterium *Chlamydia trachomatis* was present in the genital tracts of men and women experiencing urethritis and cervicitis. [19] In 1975, Holmes *et al*. published their finding from a case-control study that a significant association existed between *C. trachomatis* and NGU. *C. trachomatis* is now recognized as the most common cause of urethritis in men and of cervicitis in women and is the most frequently diagnosed STI in the world.

There are several other recognized infectious etiologies of NGU. The most prevalent of these is *Mycoplasma genitalium,* a more recently identified urogenital pathogen that accounts for 15% - 25% of NGU cases. *M. genitalium* was first isolated from the genital tracts of men with NGU in 1980 but is still considered an emerging infectious disease as its prevalence remains poorly understood; testing for *M. genitalium* is still inconsistent across clinical settings. [63] *M. genitalium* has demonstrated the

ability to acquire antibiotic resistance to the CDC-recommended first-line macrolide antibiotic Azithromycin and is therefore a growing cause for concern among health professionals. [36] *Trichomonas vaginalis* is a protozoan parasite that accounts for 2% - 13% of NGU cases. Additional infectious etiologies, determined by significant association in controlled studies, include *Neisseria meningitidis*, adenovirus, HSV-1 and 2, and very rarely *Treponema pallidum* or enteric bacteria. HSV and adenovirus are associated with a male sex partner. [6] All together, these pathogens account for between 45%-75% of male urethritis cases. The remaining percentage of male urethritis cases – up to 50% in some settings – are attributed to idiopathic urethritis (IU), a form of NGU in which cases meet the diagnostic criteria of urethritis but are pathogen-negative.

Several risk factors and sexual behaviors are traditionally associated with male urethritis, though incidence and prevalence rates may vary from population to population. In the United States, the most important risk factors associated with male urethritis due to an STI are membership in the age group of 19-24 years, African American race, low socioeconomic status, and practice of high-risk sexual behaviors. These behaviors include unprotected insertive vaginal or anal sex, unprotected sex with a new partner, multiple concurrent sex partners, and anonymous sex. [82, 84] Rates of *N. gonorrhoeae* diagnosis have steadily increased over the past decade in both men and women, but the highest rate by population is in young African American men who have sex with men (MSM). Though *C. trachomatis* is more likely to be diagnosed in men who have sex with women (MSW) than MSM, the highest rates of diagnosis occur among African American individuals, who are 5.6 times more likely than White Americans to be diagnosed. [12]

Perhaps at greatest risk from male urethritis are female partners of infected individuals. Asymptomatic inflammation and complications from urethritis in men are rare, but STI pathogens that cause male urethritis, particularly *C. trachomatis, N. gonorrhoeae,* and *M. genitalium*, are associated with severe negative clinical outcomes in women. [16] Both *N. gonorrhoeae* and *C. trachomatis* are capable of establishing asymptomatic or subclinical infection of the upper genital tract, which may lead to permanent damage of the fallopian tubes and uterus. Pelvic inflammatory disease, a complication of infection by STI pathogens in women, is associated with infertility and ectopic pregnancy. [16, 25]

1.2 Demographic Characterization of IU

IU is not distinguishable from other cases of NGU by symptoms, but there is evidence to suggest that men with IU have a distinct sociodemographic and sexual behavior profile when compared to men with pathogen positive NGU or gonococcal urethritis.

In 2006, Bradshaw *et al.* conducted the first case-control study on men with NGU to use a comprehensive set of PCR primers to detect the urogenital pathogens present in FVU samples from their patient group. The microbiota in urethral swabs and FVU samples have been shown to be highly similar and are standard in representation of the urethral microbiome. While pathogen positive NGU was associated with unprotected vaginal sex and sex with a casual partner, IU was significantly associated with insertive oral sex with a casual partner. Exclusion of men practicing unprotected vaginal or anal sex strengthened the association of IU with insertive oral sex. A significant association between IU and unprotected oral sex was reported by an earlier study in 1997 on MSM,

but they tested exclusively for *N. gonorrhoeae, C. trachomatis*, and HIV, which did not provide coverage for other known urogenital pathogens and reduced the relevancy of their findings.

A major study conducted in 2009 by Wetmore, et al., drawing information from a national health survey of young men ages 18-27, found that men with IU practicing insertive vaginal sex had fewer lifetime sexual partners than MSW without urethritis and that they practiced more consistent condom usage. They did not find an association between IU and African American or Native American/Alaskan race, which is a departure from traditional risk factors for male urethritis. Additionally, recent use of healthcare services was associated with IU but inversely associated with pathogen positive NGU. A study by the same group in 2011 again found that men with IU were significantly less likely to report African American race and significantly older than men with pathogen positive NGU. Additionally, men with IU reported fewer recent sexual partners than men with pathogen positive NGU. In 2014, Rane *et al.* reported that men with IU were twice as likely as men with *C. trachomatis* or *M. genitalium* to report a male sex partner within the past three months and were significantly more likely to report low-risk sexual practices like protected vaginal or anal sex or sex practices other than vaginal or anal sex as sole exposures.

In summary, men with IU report both fewer recent and fewer lifetime sexual partners than men with pathogen positive NGU. They are more likely to practice low-risk sexual behaviors, including insertive oral sex as sole exposure or consistent condom use. They are significantly less likely to report African American or Alaskan/Native American race than men with pathogen positive NGU. Finally, they are typically composed of an

older age demographic. These demographic characteristics clearly set men with IU apart from traditional STI patients.

1.3 Potential Etiologies of IU

It is possible that one or more unrecognized pathogens is causing IU in men. It is well established that bacterial pathogens can be transmitted from the male urethra to sexual partners and cause disease. Identifying these pathogens will allow for determination of appropriate treatment and need for partner notification.

Several bacterial species have been investigated for their role in male urethritis. Bacterial Vaginosis (BV) associated bacteria like *Atopobium* species, *Gardnerella vaginalis*, *Sneathia amnii*, BVAB 1-3, and *Megasphaera* species have been subject to close scrutiny. BV is a common condition in women characterized by a vaginal dysbiosis wherein the dominant resident *Lactobacilli* are replaced by gram-negative rods. [25, 26, 29] BV associated bacteria are often detected in the male urethra. Among this group only *S. amnii* has been found significantly more often in men with urethritis than controls and it was rarely detected without the presence of at least one other BV associated species. [52] None of the other BV associated bacteria have been found to be linked to urethritis. There are sporadic reports of male urethritis due to *Haemophilus influenzae* and *parainfluenzae*, *Streptococcus* species, and *Coynebacterium* species. No controlled, test-to-cure study has found significant associations between urethritis and these organisms. [27, 28, 8, 13, 26, 57, 17, 37, 40]

1.4 Capturing Bacterial Diversity in the Urethra

It has been appreciated that the urethra in healthy men and in men with urethritis supports a diverse community of bacteria since the mid-1960s. Investigators employing

classical cultivation and laboratory phenotyping techniques observed multiple taxa including *Corynebacterium, Streptococcus, Staphylococcus, Prevotella,* etc. Though sequencing advancements have rendered many of the classifications from these early studies defunct, they revealed that highly diverse microbiota colonized the male urethra. [4, 54]

Classical cultivation is limited by the growth requirements of fastidious bacteria, which need more complicated and expensive media and environments to establish colonies. It is estimated that only 1% - 2% of all bacterial species can be grown in laboratory conditions. [71] Phenotyping schema are often imprecise and impractical, particularly in a clinical setting. For these reasons, culture projects for the purposes of urethral microbiome surveys and pathogen discovery have largely been abandoned in favor of advanced sequencing techniques like 16S rRNA PCR and shotgun metagenomic sequencing.

The 16S rRNA gene is vertically inherited and highly conserved across all bacterial taxa. There are often multiple copies of the 16S rRNA allele in a single bacterium. The 16S PCR technique targets some portion of the allele that includes one or more of the V1-V9 variable regions. These sections are like a fingerprint and allow investigators to identify bacteria with genus-species level resolution. 16S rRNA PCR is not culture-dependent and it can be used to identify bacteria at a high-resolution with relatively minimal genetic information on hand. The technique specifically targets the bacterial 16S allele, so there is a low risk of interference from any host eukaryotic sequences present in the sample. This technique is limited by the inability of a broad range 16S primer to provide truly universal coverage and investigators may inadvertently

introduce primer bias by selecting primers that miss major taxonomic groups. 16S PCR is also limited by the completeness of the corresponding reference database.

Shotgun sequencing is a next generation sequencing technique that allows investigators to detect and sequence all genetic information in a complex sample. The datasets generated by shotgun sequencing runs are rich and can provide insight into not only the composition of microbial communities, but the activity and function of microorganisms present in the sample. Shotgun sequencing achieves a higher resolution than 16S allele sequencing, eliminates the risk of primer bias, and provides cross-domain coverage. [77] There are two key disadvantages to shotgun sequencing. High sensitivity can be problematic because host or contaminant genetic information will be sequenced along with the query sequences. These uninformative sequences may be difficult to parse out and will complicate analysis. The other disadvantage of shotgun sequencing is that the whole-genome sequences available relative to the actual taxanomic diversity of bacteria are exceptionally limited. Corresponding reference genomes are critical in making sense of shotgun metagenomic datasets because detected sequences can originate from any section of a bacterial genome and if the target strain is represented only by a 16S allele or lone gene fragment, the query sequence will be uninterpretable.

This issue is illustrated by EZBioCloud's summary of the diversity of their genome collection. The EZBioCloud reference database houses 186,000 sequenced genomes. Nearly half of those genomes are representatives of only six genera: *Escherichia, Salmonella, Streptococcus, Staphylococcus, Klebsiella,* and *Pseudomonas.* All but fifty of the 19,561 genomes of *Salmonella* are of a single species, *S. enterica*. [87] When coverage of bacterial diversity by 16S PCR alleles is compared to coverage by

whole genome sequences, it becomes clear that there are not representative genomes for major taxanomic groups of bacteria. This deficit restricts the ability of investigators to make use of metagenomic shotgun datasets because the absence of reference sequences can skew analysis to misrepresent the taxonomic composition of the sample.

The utility of 16S rRNA PCR and metagenomic shotgun sequencing is limited by one additional factor intrinsic to culture-independent sequencing methods. The detection of sequences in a sample requires only that the genetic information is present at time of sampling. These methods are therefore incapable of distinguishing between incident contamination and live bacteria in environmental samples. In an environment like the distal urethra of a sexually active male, sources of contamination are abundant.

1.5 Research Objectives

We were interested in identifying pathogens that may cause IU using multiple methods. To this end, we undertook the Idiopathic Urethritis Men's Project (IUMP), a longitudinal case-control study of men with IU based in Indianapolis, IN.

We generated metagenomic and 16S datasets from the DNA extracted from urethral swabs of men with NGU, which provided comprehensive detection and sequencing of the genetic information present in the samples. In order to address the previously discussed disadvantages of these sequencing techniques I initiated extensive cultivation of bacteria from the paired FVU samples collected from men with IU.

I hypothesize that using various growth conditions to culture bacteria from the FVU samples of men with IU will yield previously uncultured clinical isolates of fastidious bacteria from IU specimens that may be pathogenic. I further hypothesize that

genome sequencing of novel isolates will increase database coverage of the male urethral microbiome and improve understanding of its composition in health and disease.

Methods and Materials

2.1 Study Design

The IUMP recruits and enrolls study participants at the Bell Flower Clinic, a public clinic for STI diagnosis and treatment in Indianapolis, IN. The Bell Flower Clinic operates as part of the Marion County Public Health Department's STD Program. The clinic sees individuals 14 years and older, Monday through Friday, on an appointment or walk-in basis. They offer testing for gonorrhea, chlamydia, syphilis, and HIV, exams for vaginal and urethral symptoms, and treatment for most STI.

To be eligible as a urethritis patient in the IUMP, an individual must be symptomatic and show microscopic evidence of inflammation. Rather than the diagnostic standard of 2 PMNLs or more, potential participants must have 5 PMNLs or more per high powered field on a gram stain of a urethral swab. Patient specimens were tested for *N. gonorrhoeae, C. trachomatis, M. genitalium, T. vaginalis*, and *U. urealyticum*. Testing for pathogens was carried out by the Indiana University School of Medicine Infectious Disease laboratory. Additionally, cases must not have a history of antibiotic use within the last thirty days.

Patients make at least two visits to the Bell Flower Clinic. The first visit is when they are recruited, enrolled, and provide baseline samples. They provide a detailed medical and sexual history, undergo the clinical exam, and provide an FVU sample and two urethral swabs. The urine sample is used for pathogen testing and bacterial culture. The first of the urethral swabs is used for metagenomic sequencing, and the second is stored at -80ºC. Patients in the urethritis group receive treatment at the baseline visit and are asked back for a follow-up visit in one month. At the follow-up visit, they again

provide a detailed medical history, undergo a clinical exam, and provide a new FVU sample and two additional urethral swabs for NAAT testing and metagenomics sequencing. IUMP enrollment is ongoing, though the present study accounts for 216 urethritis patients.

The Institutional Review Board for Indiana University School of Medicine approved this study. All participants provided written informed consent prior to enrollment in the IUMP.

2.2 Five Pathogen Testing

The Indiana University School of Medicine Infectious Diseases Research Laboratory conducted testing on FVU samples for *N. gonorrhoeae, C. trachomatis, M. genitalium, T. vaginalis* and *U. urealyticum*. NAAT testing for *C. trachomatis* and *N. gonorrhoeae* was performed as previously described. [44] *T. vaginalis*, *M. genitalium*, and *U. urealyticum* NAAT testing was performed using quantitative PCR (qPCR) assays developed in-house as previously reported. [45] Symptomatic men negative for all five pathogens were considered to have IU.

2.3 Culture Methods

All FVU samples from the 59 patients who met the criteria for IU were plated on four types of solid media for culture of bacterial isolates. I used chocolate agar plates (CAPs), BD BBL™ MacConkey agar plates, BD BBL™ Columbia SB agar plates, and BD CDC anaerobe™ agar plates (Sparks, MD). The CAPs were either BD Chocolate II™ Agar or produced in-house. Briefly, components of CAPs were 10 g bovine hemoglobin, 36 g of Difco™ GC Medium Base, and 10 ml of Isovitalex diluted in 1 L of autoclaved Millipore-filtered water. All plated FVU samples were incubated at 35º C for

five days in anaerobic (1% or less O_2) or microaerophilic (5% -10% O_2) environments. The environments were generated using the Anaerobic and Campy BD GasPak™ EZ Gas Generating Systems according to manufacturer instructions (Sparks, MD). At the end of the five-day incubation period, isolated colonies were harvested from the agar plates and transferred into 500µl of 15% glycerol BD Tryptic Soy Broth for storage at -80ºC (Sparks, MD). A small portion of the broth containing the isolated colony was kept separate for 16S rRNA polymerase chain reaction (PCR).

2.4 rRNA 16S Allele PCR and Strain Identification

Individual isolated colonies in 15% glycerol BD Tryptic broth or autoclaved Millipore-filtered water were boiled for ten minutes to lyse the cells and then prepared for PCR cycling. PCR conditions were optimized for a 25 µl per sample reaction mix with the Thermo-Fisher Phusion™ High-Fidelity DNA *taq* polymerase (Waltham, MA). All bacterial strains were assessed at least once with the 1492R/27F broad-range primer set, which targets the full-length 16S allele (Table 1). Any isolates failing to amplify were assessed a second time using more sample with 1492R/27F.

Primer Name	Oligonucleotide Sequence	Target
DN069 (1492R)	5' - TACGGNTACCTTGTTACGACTT - 3'	16S rRNA gene
DN070 (27F)	5' - AGAGTTTGATCATGGCTCAG - 3'	16S rRNA gene
GVAG1F (Fwd-Gar)	5' - TTCGATTCTGGCTCAGG - 3'	16S rRNA gene
GVAG1R (Rev-Gar)	5' - CCATCCCAAAAGGGTTAGG - 3'	16S rRNA gene

Table 1. 16S rRNA Primers Summary of the oligonucleotide sequences and targets of the two 16S primer sets. [38, 49]

I noticed the absence of *Gardnerella* spp. isolates from the initial rounds of PCR, which was surprising because sequences corresponding to these organisms were abundant in the parallel metagenomic data set. I chose to assess all strains failing to amplify with the broad-range primer set using primers optimized for *Gardnerella* spp. (Table 1). Any strains failing to amplify in this third round were assessed a fourth and final time with the *Gardnerella* spp. primer set. Any strains failing to amplify in the fourth round of PCR were abandoned. PCR products were evaluated for amplification and appropriate band length using gel electrophoresis on a 1% agar (Figure 1). The bacterial 16S rDNA allele is 1.5 kb in length.

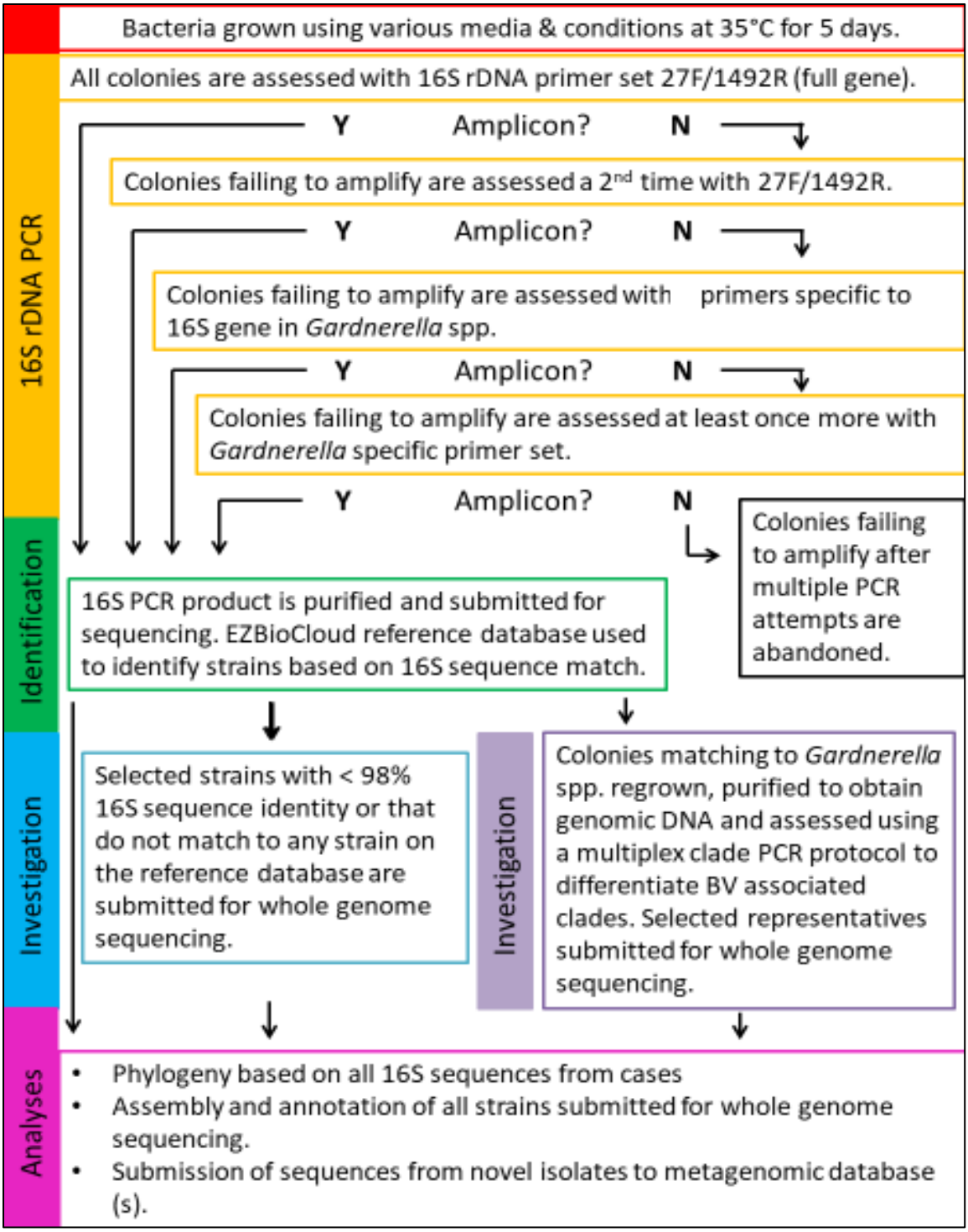


Figure 1. **Strain Sequencing and Identification Workflow** Workflow for 16S PCR and strain identification for the present study along with downstream research goals.

I used Eurofin Genomics (Louisville, KY) to sequence the PCR products by Sanger sequencing and the EZBioCloud 16S database to identify the bacterial isolates. EZBioCloud is strictly curated database containing the taxonomic hierarchy of Bacteria and Archaea. All submissions are screened for quality and carefully categorized to preserve the integrity of the bioinformatics analyses conducted by Chun Labs (South Korea), the group that operates EZBioCloud. [87] Based on percent sequence similarity/identity, I categorized isolates as a species match at ≥ 98% sequence similarity and as a genus match at ≥ 95% sequence similarity to the nearest type strain. If an isolate did not have ≥ 95% sequence similarity to any taxon, I recorded the closest type strain. If strains had 0% sequence similarity according to EZBioCloud, I categorized them as No Hit.

2.5 Multiplex Clade PCR

I tested isolates identified as *Gardnerella* to determine if they were members of four previously described and clinically relevant clades within the genus. [1] *Gardnerella* isolates were regrown on CAPs in an anaerobic environment. Purified genomic DNA was obtained using the IBI Scientific gMAX Genomic DNA Mini Kit (Dubuque, IA) according to manufacturer instructions, with an extended 1 hr cell lysis period at 60ºC. I used a PCR protocol optimized for the TaKaRa Ex *Taq* DNA polymerase (Japan) from the laboratory of Amanda Lewis (Washington University, St. Louis) to determine clade membership. Clade-specific primer sets targeted genes exclusive to each of the four clades. A fifth primer set acted as a control, targeting a gene common to all strains of *Gardnerella* (Table 2). PCR products were evaluated using gel electrophoresis on a 1%

agar gel. Each lane was assessed for the presence of two bands corresponding to the control gene and clade specific marker.

Primer Name	Oligonucleotide Sequence	Target
Gvag *tuf* F1 (203)	5' - ATGGCAAAGGAAAAGTACGAGCG - 3'	*Tuf* gene
Gvag *tuf* R1 (204)	5' - TCAAGCCCTCTTCCATTGCG - 3'	
Gv alpha *fuc* F4 (212)	5' - CAGATGCTGTTATCAGTGTTTGC - 3'	Clade 1
Gv alpha *fuc* R4 (213)	5' - GTAGACGCAAATTTGCGAGAG - 3'	
B513 hypoth F (195)	5' - GCGACCTTGTAACTATAAGTTCCCAG - 3'	Clade 2
B513 hypoth R (196)	5' - CGTATGGACTAGAATCGTCTTGTGGA - 3'	
B483 thioredoxin F (197)	5' - GGCAGTTATTCACGCAAGCC - 3'	Clade 3
B483 thioredoxin R (198)	5' - AGTCTTCTGAATCGTCGTCCGT - 3'	
409-05 chloride F (199)	5' - CAGTTATGGCTGGGGTTGGT - 3'	Clade 4
409-05 chloride R (200)	5' - CTAAAGCTCGACCATTGCCCA - 3'	

Table 2. Multiplex Primers The multiplex clade PCR uses five total primer sets per reaction mixture. The *tuf* gene targets all strains of *Gardnerella.* The sequences and specific gene targets for all five of the primer sets are summarized in the table.

Results

3.1 NGU Case Demographics

Of 216 patients enrolled, 70 men were positive for *C. trachomatis*, 36 were positive for *M. genitalium*, 17 were positive for *U. urealyticum*, and 6 were positive for *T. vaginalis*. 28 men were positive for two or more of these pathogens. The remaining 59 men were negative for any of these STI pathogens. These were the IU patients (Figure 2). To determine if the IU cohort reflected the findings in previous studies that men with IU are characteristically divergent from traditional STI demographics, I compared all cases positive for at least one of the traditional STI five pathogens with the IU group.

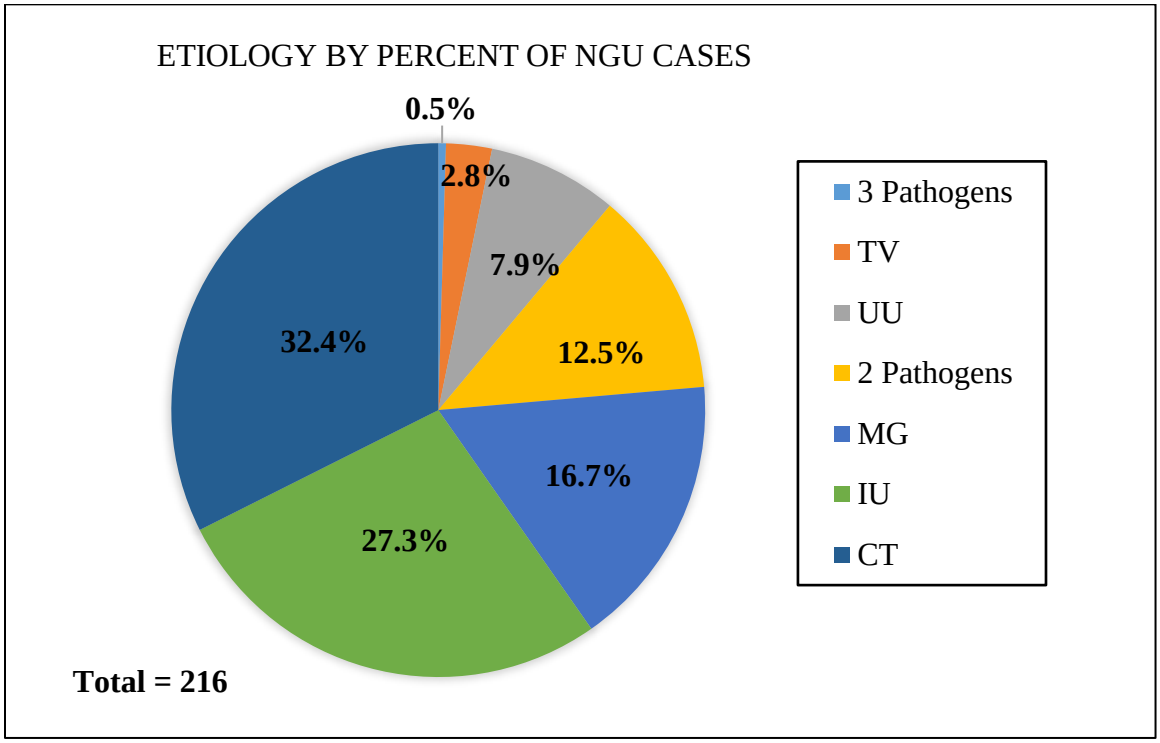


Figure 2. Etiology by Percent Percentages of NGU cases due to various etiologies based on five pathogen testing. CT is *C. trachomatis*, TV is *T. vaginalis*, UU is *U. urealyticum*, MG is *M. genitalium*, and IU is idiopathic urethritis. Two pathogens were found in 12.5% of the cohort (N=27) and three pathogens were detected in 0.5% (N=1).

Based on previous findings in comparisons between IU patients and other STI groups, I expected to find that IU was associated with an older median age, Caucasian race, and particular sexual behaviors like insertive anal or oral sex. [6, 81, 83] When I compared the patient group with IU to the pathogen positive NGU patient group, I found that there were no significant differences between them in age, race, or previous diagnoses of traditional STI including *C. trachomatis, N. gonorrhoeae,* and *T. vaginalis*. There was no significant difference in rates of Received Oral Sex within the past 60 days. The patient groups with pathogen positive NGU and with IU were also similar in the rates of self-reported ethnicity and membership to a race other than African American or Caucasian American. (Table 3) The key differences between the two patient groups were in the categories of self-reported sexual orientation and self-reported sexual behaviors. Men in the IU patient group were significantly more likely to self-identify as MSM – IU patients indicated choice of a male sex partner more than twice as often as men in the pathogen positive NGU group. Men with IU were also significantly more likely to report insertive anal sex within the past 60 days ($p = <0.0001$) and were significantly less likely to report insertive vaginal sex within the past 60 days ($p =0.003$) than men with pathogen positive NGU. Additionally, men with IU more likely to self-report a history of a non-traditional STI diagnosis ($p = 0.05$) than men with pathogen positive NGU. (Table 3)

Characteristic	Pathogen + (n=156)	IU (n=59)
Age, median (range)	27 (18-61)	31 (20-64)
Race, n (%)		
African American	98 (62.8)	43 (72.9)
Caucasian	34 (21.8)	7 (11.9)
Other	24 (15.4)	9 (15.3)
Ethnicity, n (%)		
Hispanic	6 (3.8)	2 (3.4)
Non-Hispanic	150 (96.2)	57 (96.6)
Sexual Orientation, n (%)		
Homosexual (MSM)	10 (6.4)	12 (20.3)*
Heterosexual (MSW)	141 (90.4)	45 (76.3)
Reported Previous STI Diagnosis, n (%)		
Chlamydia	82 (52.6)	35 (59.3)
Gonorrhea	55 (35.3)	27 (45.8)
Trichomoniasis	17 (10.9)	10 (16.9)
Non-specific NGU, other	58 (37.2)	32 (54.2)**
Reported Sexual Behaviors		
Lifetime partners, median (range)	10.5 (1-176)[a]	10 (4-200)
Partners, past 60 days, median (range)**	2 (0-20)	2 (1-11)[b]
Received Oral Sex, past 60 days (y/n) (y%)	124/32 (79.5)	58/1 (98.3)
Insertive Anal Sex, past 60 days (y/n) (y%)	25/131 (16.0)	38/21 (64.4)***
Vaginal Sex, past 60 Days (y/n) (y%)	129/27 (82.7)	52/7 (88.1)

Table 3. Demographics Summary of the demographic, medical, and behavioral characteristics of NGU cases 001-216. [a] 60 participants indicated an unknown number of lifetime partners [b] 4 participants did not provide an answer.

Rank-sum analysis was used for comparison of medians. Chi-square testing was used for comparison of groups.

* $p = 0.003$

** $p = 0.05$

*** $p = <0.0001$

These findings were consistent with previous studies in that men with IU were more likely to self-identify as MSM and much more likely to report practicing insertive anal sex. [35, 83] Participants were asked to indicate how often they used condoms during insertive vaginal and anal sex by reporting how many times they used a condom out of their last ten sexual encounters. I categorized answers (0 – 3)/10 as rarely/never, (4-6)/10 as occasionally, and (7-10)/10 as consistent condom use. Based on previous findings, I expected the IU group to report lower risk sexual practices than the pathogen positive group, but there was no difference between the groups in condom use for insertive vaginal or anal sex (data not shown). [82, 84] When asked about condom use for insertive oral sex, both groups reported that they were much more likely to practice unprotected insertive oral sex than wear a condom during those encounters.

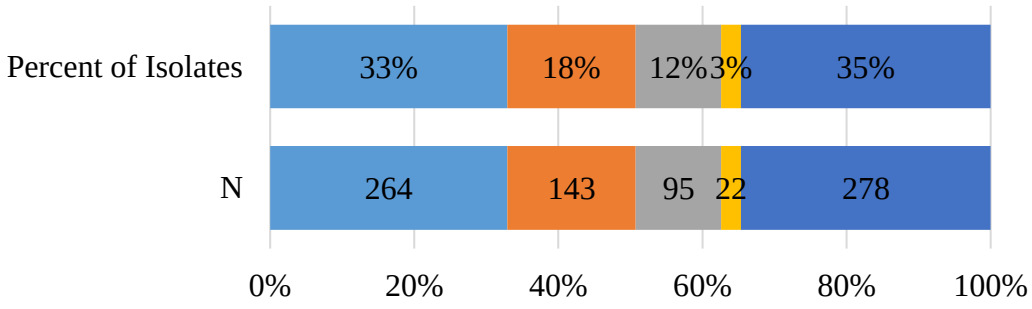
To summarize, these findings diverge somewhat from the literature in that I found no associations between IU and receiving oral sex, more consistent condom usage, higher median age, or membership to a race other than African American. Comparison between the IU patient group and the pathogen positive NGU group does indicate, however, that IU is associated with MSM sexual orientation and insertive anal sex as well as a history of STI other than gonorrhea, chlamydia, or trichomoniasis. These associations are more consistent with previous studies. [7, 12, 19, 51, 55]

3.2 The IUMP Isolate Collection

We plated FVU samples from each of the 59 IU patients provided at their baseline visit on the described growth conditions. Seven of these samples did not produce any isolates on any of the media. The remaining 52 patient samples produced at least one bacterial isolate. The number of isolates per sample was highly variable, ranging from

only one isolate over all of the media types to 42 isolates from a single FVU sample. We attempted to identify each isolate individually using one or more rounds of 16S rRNA PCR and successfully generated and sequenced 524 PCR amplicons, representing 65.3% of isolates in the collection (sequences of 300 bp or less were discarded).

Using the EZBioCloud database, I identified 264 isolates at the species level (≤ 98%). With the additional 143 isolates identified to at least the genus level, I identified a total of 407 isolates to at least the genus level (≤ 95%). [43] 95 of the isolates fell below the genus level cutoff but bore some percent sequence similarity to a type strain on EZBioCloud. 22 of the isolates did not match to any sequence on EZBioCloud, which I confirmed using the NCBI BlastN bacterial 16S database (Figure 3). [87]



	N	Percent of Isolates
Species (≥ 98%)	264	33%
Genus (98% >, ≥ 95%)	143	18%
Closest Match (> 95%)	95	12%
No Hit (0%)	22	3%
No Amplicon	278	35%

Figure 3. Categorization of Isolates by Percent Sequence Similarity Summary of all isolates by category based on present sequence similarity to a type strain on the EZBioCloud database. No Amplicon indicates the 278 isolates that did not produce an amplicon during 16S PCR. The total number of isolates is 802.

Considering our goal of identifying novel and potentially pathogenic bacterial strains, we have generated a substantial pool of isolates to investigate further with 67% of the isolate collection at lower than species level similarity to a type strain on the EZBioCloud database.

There was a high degree of variability in both the number of isolates and the number of genera per patient FVU sample. The average number of isolates per patient sample was 17.5 with a median of 18 (1-42) (Figure 4a). The average number of genera per patient sample was 3.25 with a median of 3 (1-7) (Figure 4b). While I expected that the number of genera per sample would consistently increase with the number of isolates per patient sample, I found that the relationship between these two variables was weak (Figure 4c). This is likely because high productivity is not necessarily indicative of high diversity. For example, one of the patient samples produced 22 isolates, but they typed to only two genera. In contrast, another patient sample produced only 7 isolates, but they typed to 3 different genera (data not shown).

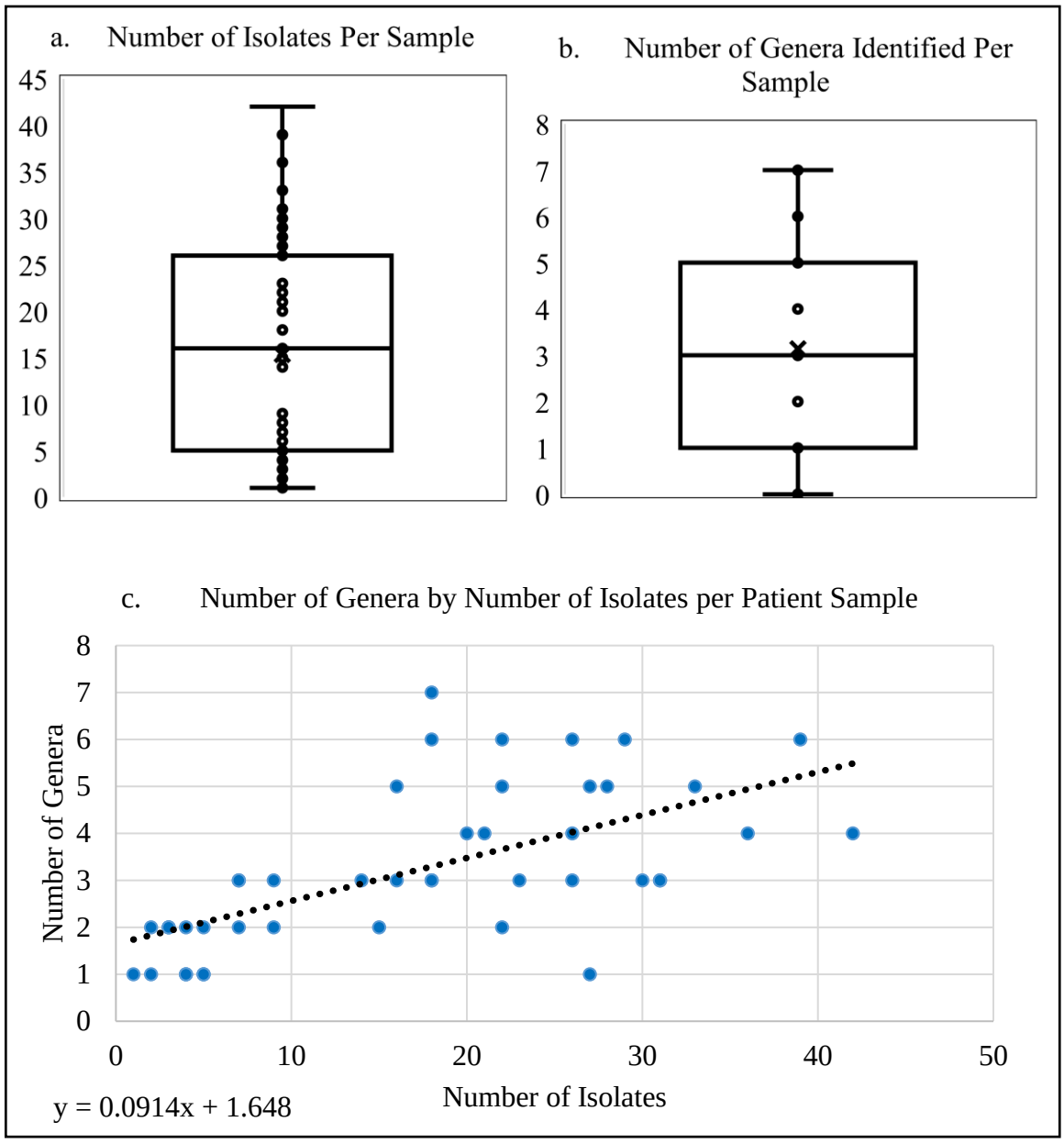


Figure 4. Descriptive Statistics for the Strain Collection a. Box-Whisker plot of the number of isolates per FVU sample. Median 18 (1 - 42), with a mean of 17.25. **b**. Box-Whisker plot of the number of genera per FVU sample. Median 3 (1-7), mean of 3.2. **c.** Linear regression of number of genera by number of isolates, $R^2 = 0.3895$.

There are 24 genera represented by the 407 isolates that I identified to at the least the genus (≥ 95%), with the top six most frequently identified genera - *Streptococcus, Staphylococcus, Haemophilus, Corynebacterium, Gardnerella,* and *Prevotella* - representing nearly 80% of the group (Figure 5). Six of the 24 genera appeared only once (Figure 5).

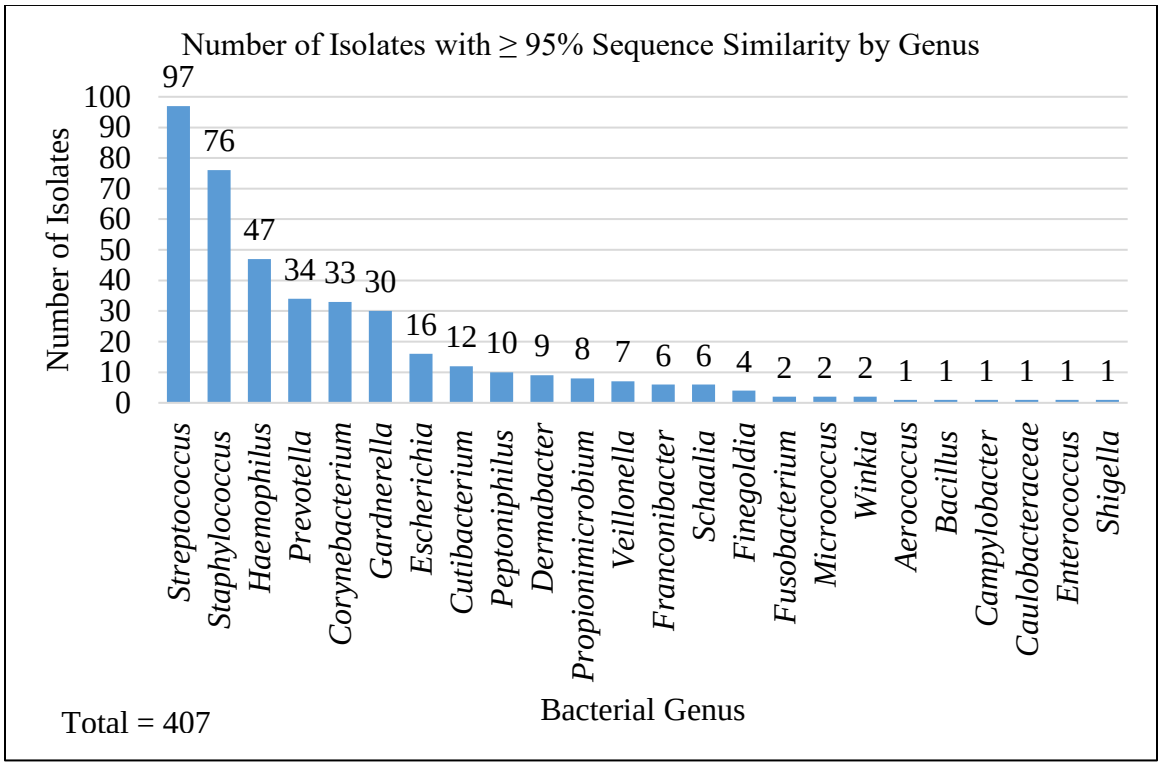


Figure 5. Number of Isolates with ≥ 95% Sequence Similarity by Genus

Bar graph describing the number of isolates per genera for all isolates with 95% or greater sequence similarity to a type strain on the EZBioCloud database. Data labels indicate number of individual isolates.

I was interested in which genera were the most common among the IU patient group. To account for the high degree of variability in sample productivity, I counted one representative of a single genus per patient, e. g. in a patient sample with 22 isolates typing to *Escherichia, Escherichia* was counted only once for that patient (Figure 6).

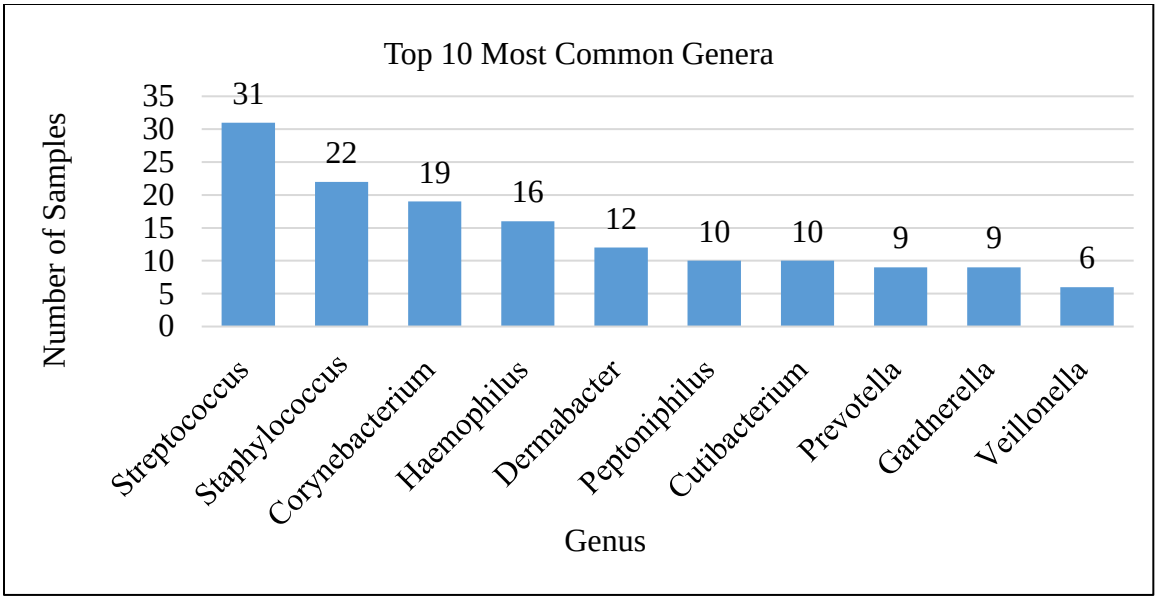


Figure 6. Top 10 Most Common Genera The top ten most common genera among IU patients.

Once I looked at the most common genera by sample, I found that *Dermabacter*, *Peptoniphilus*, and *Cutibacterium* were identified in more participants than either *Prevotella* or *Gardnerella*, which re-ordered the rankings of most abundant genera based on individual isolates (Figure 6).

To qualitatively evaluate the isolate collection, I searched for reference sequences corresponding to the isolates identified to the species level (≥ 98%) within the top six most frequently identified genera. I restricted the search to reference sequences present in the EZBioCloud database. The key findings of that search are described below.

The most abundant genus within the isolate collection was *Streptococcus*, with 69 members of *Streptococcus* identified at the species level (Figure 7). Of this group, the two most common species were the closely related *S. mitis* and *S. periodonticum*. These two species were originally isolated from the human oral cavity. *S. mitis* is frequently observed in human infections including urinary tract infections, meningitis, and infective

endocarditis. [70] There was no reference strain reported originating from the vagina or male urethra on EZBioCloud. *S. periodonticum* is a recently described species which as of yet has not been reported to cause disease and has not been published as a resident of the male urethra or vagina. [87] *S. pseudopneumoniae* and *S. oralis* group spp. were the next most common species in this group. Both are reported primarily in the oral cavity and nasopharynx. [46, 57] The *S. constellatus* spp. are also residents of the oral cavity. Only one, *S. constellatus subsp. constellatus*, had a representative genome isolated from the vagina. Of all the *Streptococcus* species identified, only *S. agalactiae*, which was identified one time only, is a documented in male urethritis cases and frequently isolated from the vagina. *S. agalactiae* is also called Group B *Streptococcus* and causes neonatal infections and infections in immunocompromised adults. [8]

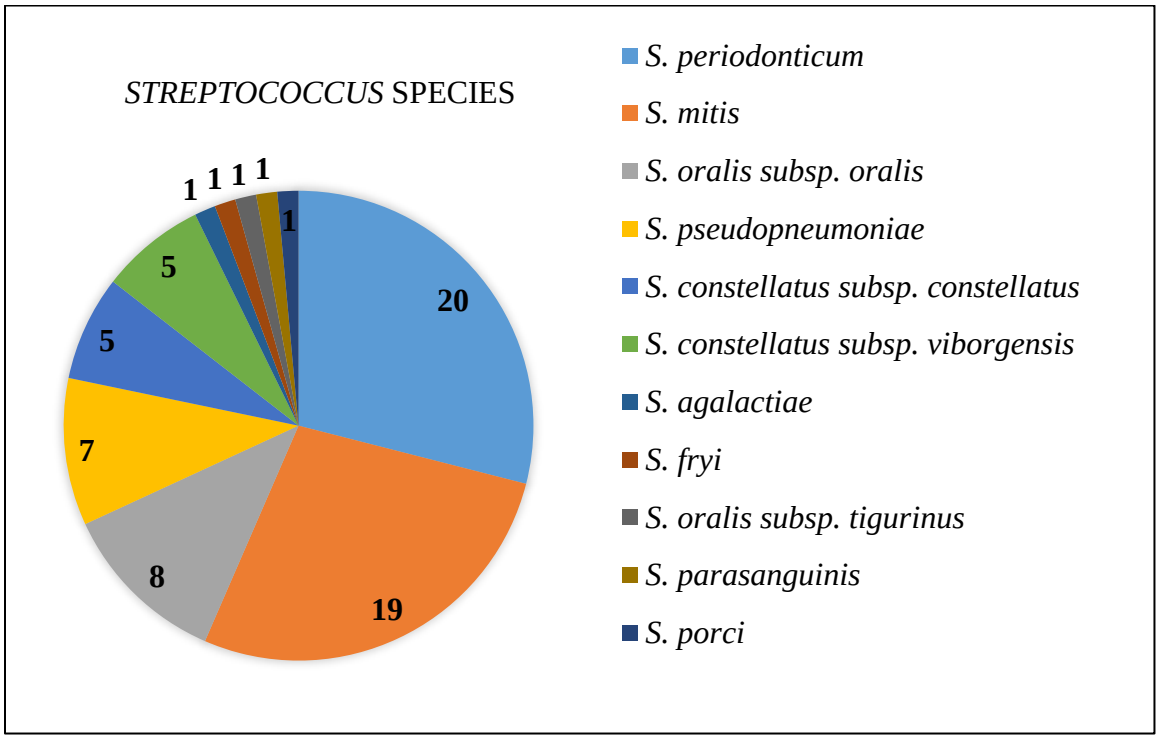


Figure 7. *Streptococcus* Isolates All identified at ≥ 98% sequence similarity to the nearest type strain. The legend is ordered from most to fewest isolates.

I identified 59 isolates as *Staphylococcus* (Figure 8). *S. epidermidis* is part of the normal skin and mucosal flora of humans. It is often observed in infections in immunocompromised and catheterized patients. There are examples of isolates from vaginal and urethral samples in the EZBioCloud reference database. [87] *S. hominis subsp. novobiosepticus* is another member of the skin flora. Normally a commensal, this bacterium has been reported in severe infection in cancer patients. There are representative genomes isolated from the urinary tract, rectum, and vagina. [87] *S. haemolyticus* is another member of the skin flora, often found in high concentrations at the perineum, and an opportunistic pathogen. [87] Of the remaining *Staphylococcus* species, *S. argenteus* alone is frequently observed in human infections. The remaining *Staphylococcus* species are all represented by genomes isolated from skin, but none originated from the male urethra or vaginal samples. [87]

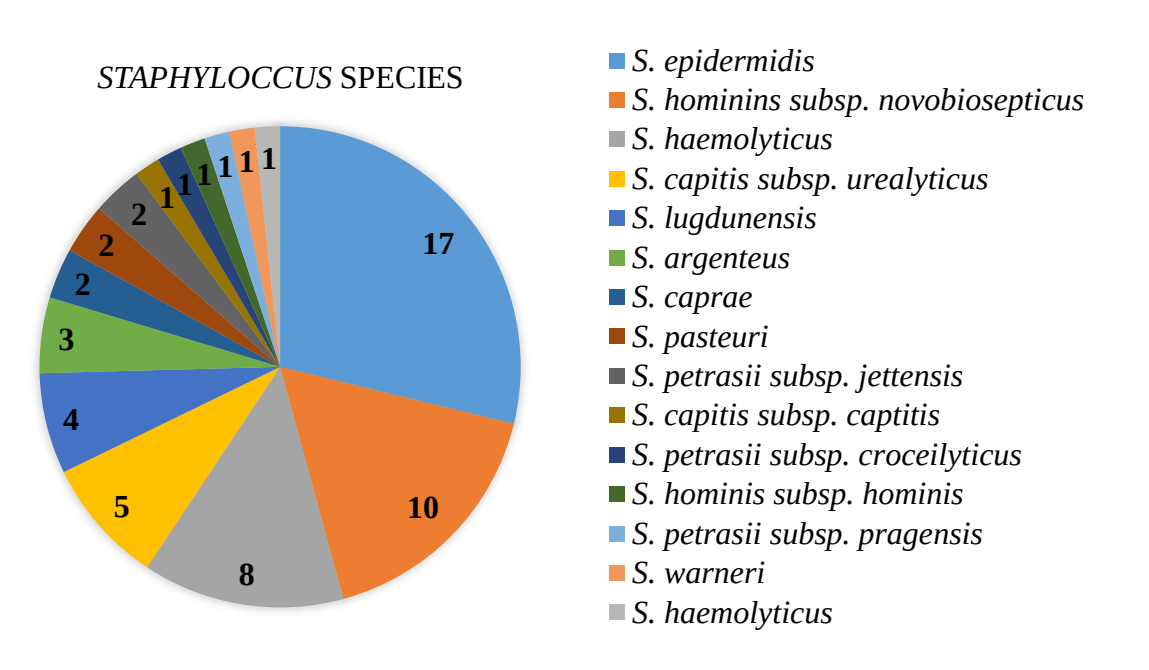


Figure 8. *Staphylococcus* Isolates All identified at ≥ 98% sequence similarity to the nearest type strain. The legend is ordered from most to fewest isolates.

Corynebacterium was the third most frequently identified genus though only 16 isolates identified as Corynebacterium spp. (Figure 9). *C. glucuronolyticum, C. simulans, C. auricomucosum, C. phoceense, C. singulare, C. amycolatum*, and *C. striatum* are documented residents of the vagina and male urethra. [87] *C. glucuronolyticum* has been reported in cases of male urethritis and prostatitis and is very closely related to *C. seminale*, a suspected pathogen of the urogenital tract in men. [27, 28]

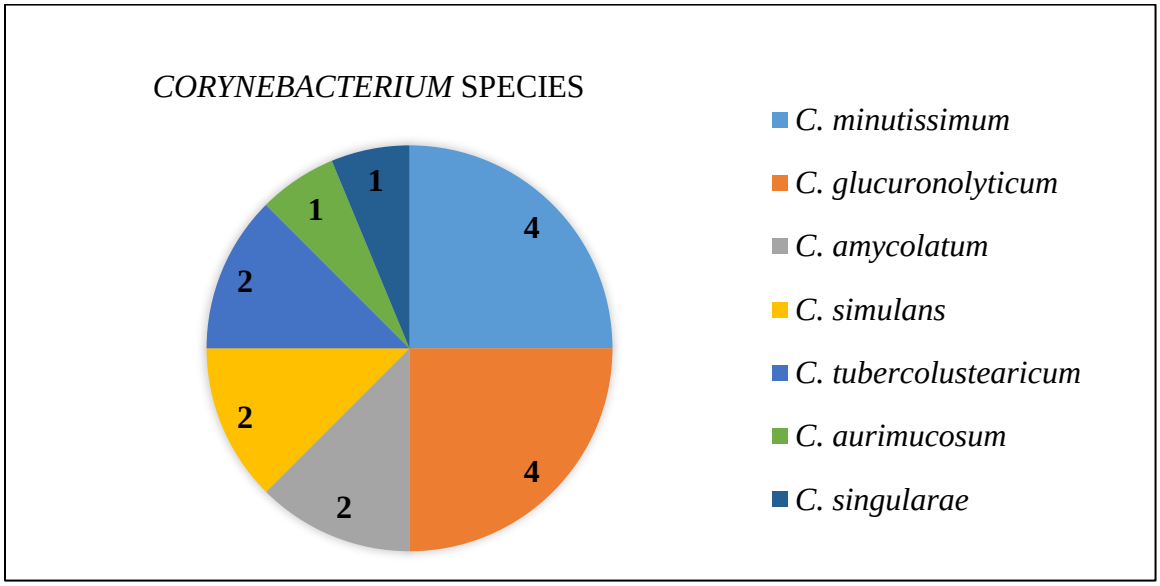


Figure 9. *Corynebacterium* Isolates All identified at ≥ 98% sequence similarity to the nearest type strain. The legend is ordered from most to fewest isolates.

20 of the isolates were identified as a species of *Prevotella* (Figure 10). These bacteria are well established as residents of the human oral cavity and female urogenital tract. [25] They have been detected in men but are represented exclusively by strains from the vagina in the EZBioCloud 16S database. [87] *P. bivia* is particularly interesting due to its potential role in BV in women. There is evidence to suggest that the interaction between *Gardnerella vaginalis* and *P. bivia* in the vaginal flora contributes to BV

pathogenesis. [29] Only two of the patient samples produced both genera; more often *Prevotella* occurred independently of *Gardnerella*.

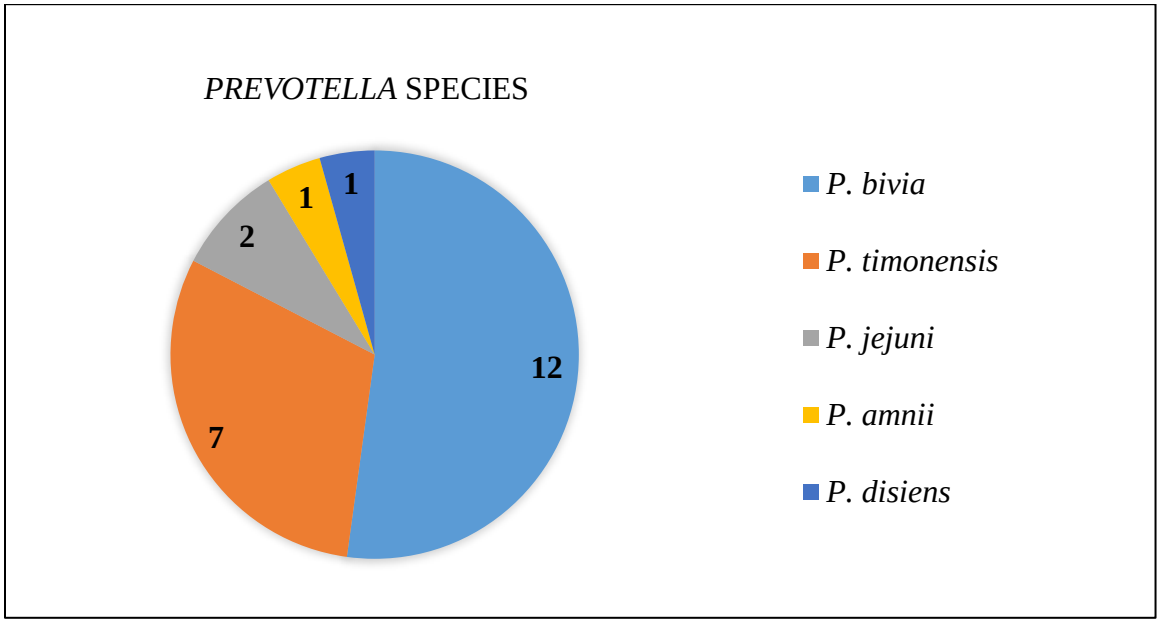


Figure 10. *Prevotella* Isolates All identified at ≥ 98% sequence similarity to the nearest type strain. The legend is ordered from most to fewest isolates.

22 isolates were identified as species of *Haemophilus* (Figure 11). There is compelling evidence to support a pathogenic role in male urethritis for these bacteria. Both *H. parainfluenzae* and *H. influenzae* have been reported in in multiple cases of male urethritis. No study has ever found a significant association, perhaps due to the low relative occurrence in most populations. [17, 34, 37] Both *H. quentini* and *H. aegyptius* were discovered as closely related species to *H. influenzae* in the male urethra. [39] In the present study, *Haemophilus* species were frequently identified in patients, but detected in only two of the 84 healthy controls samples (data not shown). Furthermore, these findings lend support to the hypothesis that IU is associated with orogenital exposure, as both *H. influenzae* and *H. parainfluenzae* colonize the oropharynx. [17, 37, 40]

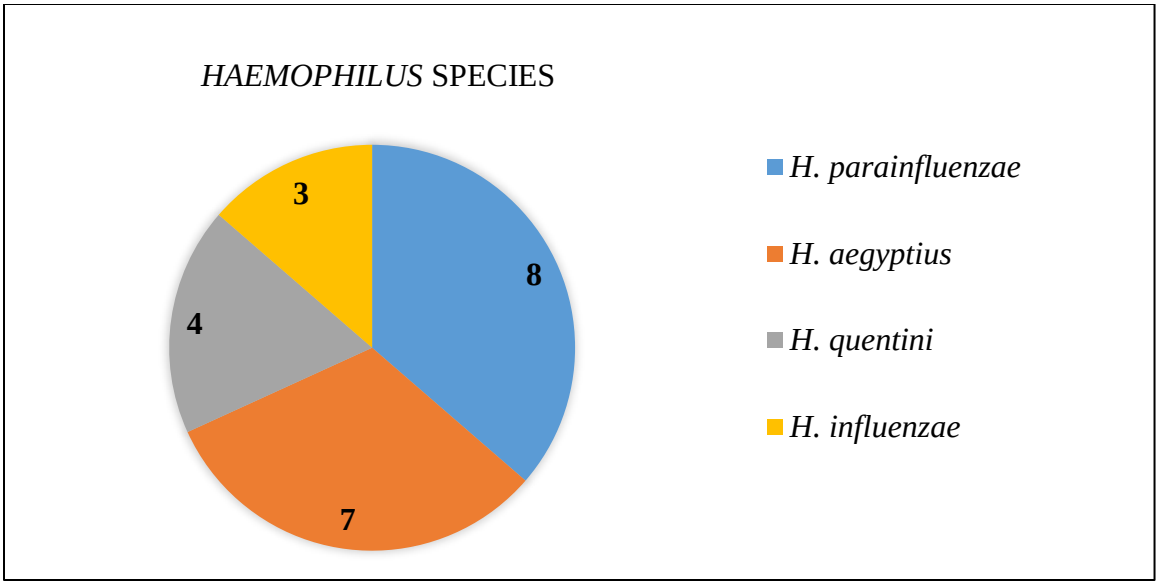


Figure 11. *Haemophilus* Isolates All identified at ≥ 98% sequence similarity to the nearest type strain. The legend is ordered from most to fewest isolates.

Members of the genus *Gardnerella* have been heavily scrutinized as potential pathogens in idiopathic urethritis due to the tight association between *Gardnerella* spp. and BV in women. *G. vaginalis* is the predominant organism detected in BV, which is one of the chief causes of vaginal complaints in women of reproductive age. [25, 26] BV is associated with multiple adverse clinical outcomes including pre-term birth, ectopic pregnancy, endometriosis, and pelvic inflammatory disease. [16] Though it appears that *G. vaginalis* is the dominant species within the genus in the isolate collection, it is possible that the distinctions between various recently described species of *Gardnerella* have not been included in the reference database (Figure 12). It is likely that we will observe more diversity identified within the genus over time. A pathogenic role in urethritis has never been established for *Gardnerella* spp.; they are detected as frequently in healthy controls as they are in urethritis cases, and it is unclear what virulence factors these bacteria possess. [24]

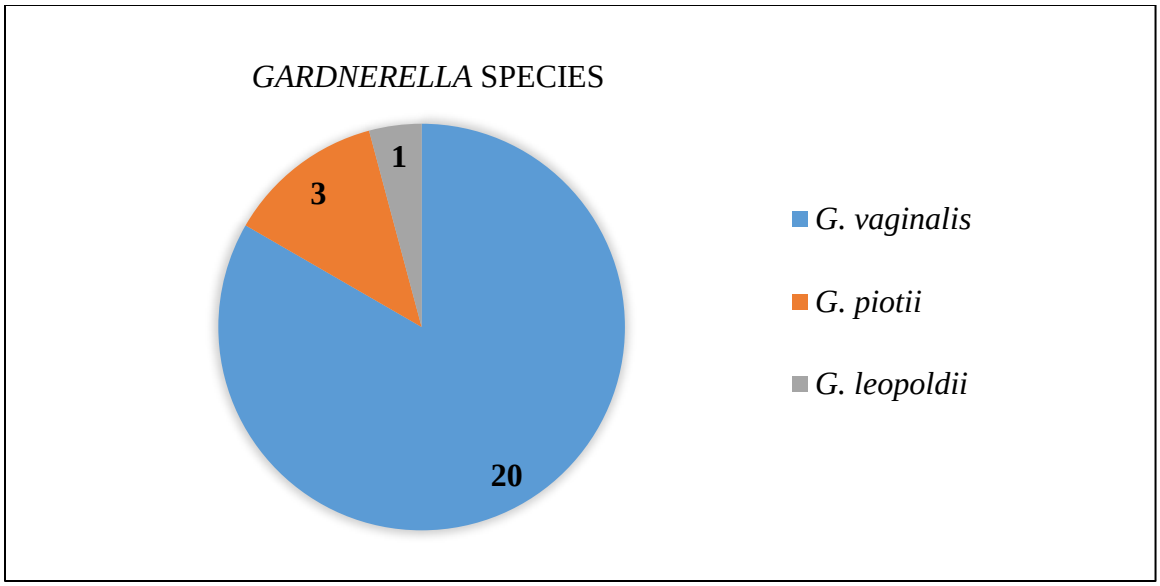


Figure 12. *Gardnerella* Isolates All identified at ≥ 98% sequence similarity to the nearest type strain. The legend is ordered from most to fewest isolates.

3.3 Multiplex Clade PCR for *Gardnerella* Isolates

In 2012, Ahmed et al., by analysis of whole genome sequences of 17 clinical isolates, determined that there were four genetically isolated clades of *G. vaginalis*. [1] Balashov et al. previously reported that two of these clades, Clade 1 and Clade 3, and that colonization by multiple clades simultaneously were associated with BV in women. [3] I applied a multiplex clade PCR to our own *Gardnerella* isolates from the IU cases and from a group of men without urethritis to determine if these participants were colonized by any of the four clades. I included this group of men without urethritis because they all had a high relative abundance of *Gardnerella* spp. in metagenomic sequencing analyses but were not experiencing any symptoms or inflammation. If *Gardnerella* is a pathogen in BV, these men without urethritis could be considered asymptomatic carriers.

I tested 19 strains from the IU cases and 35 representative isolates from asymptomatic men and identified representatives from Clade 1, Clade 2, and Clade 4

from both groups. None of the isolates typed to Clade 3. The median number of clades present in a single individual was two (data not shown). Five of the *Gardnerella* isolates, all from the same patient sample, did not type to any of the four clades (Table 4). These isolates may be representatives from the missing Clade 3 or indicate a clade of *Gardnerella* that was not detected in the original study (Table 4). They chose isolates from women exclusively and may have inadvertently excluded a clade more common in men.

	IU Patients n=19	**Asymptomatic n=35**
Clade 1	3	9
Clade 2	7	15
Clade 3	0	0
Clade 4	4	11
Unknown	5	0

Table 4. Multiplex PCR Results Distribution of genetically isolated clades of Gardnerella among two test

This is the first example of clinical isolates from IU patients and asymptomatic men typed according to these BV-associated clades. These findings show that men with and without acute urethritis can carry at least one of the BV-associated clades and be colonized with multiple clades simultaneously. If it is determined that *Gardnerella* spp. contribute to BV pathogenesis, these finding lend support to the hypothesis that BV can be sexually transmitted from male to female partners during penile-vaginal sex.

3.4 Novel Bacterial Strains

With a species designation at ≥ 98% sequence similarity to the type strain 16S allele, just under half of the existing isolate collection may represent novel bacterial species (Figure 3). The lower the percent sequence similarity is to a type strain, the greater the taxonomic distance of the query sequence. I was interested in how strains that bore < 95% sequence similarity to a type strain were distributed by distance from the most similar type strain (Figure 13). When I categorized the 95 isolates in this group by percent sequence similarity, the bulk of their 16S allele sequences fell between 90% - 95% percent similarity to the closest type strain on EZBioCloud.

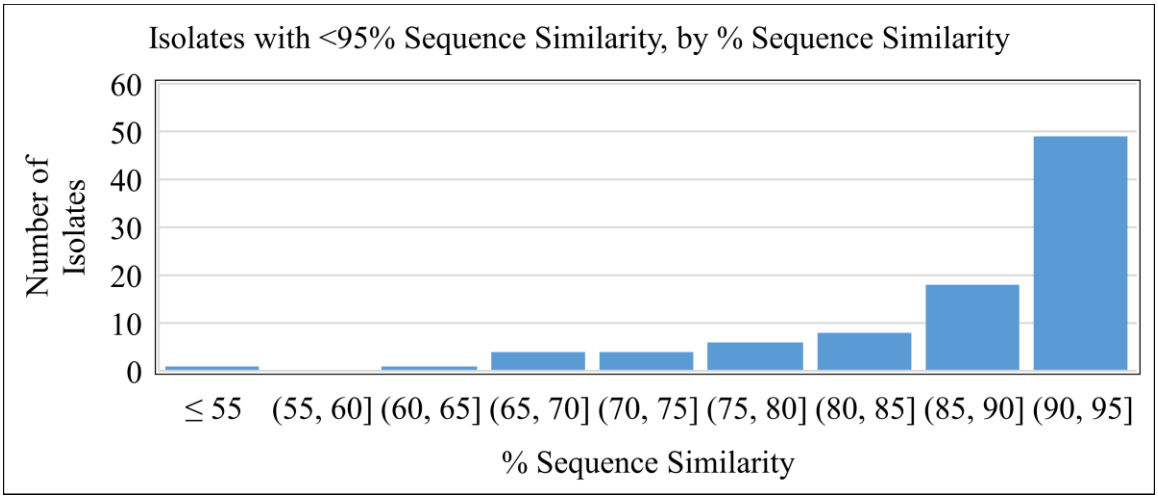


Figure 13. Isolates with <95% Sequence Similarity, by % Sequence Similarity

95 strains with < 95% sequence similarity to an existing in the reference database categorized by 5% intervals from 55% - 95%.

Discussion

I conducted an extensive culture project as an approach to pathogen discovery in idiopathic urethritis. The IUMP isolate collection was cultivated with the purpose of supporting and enriching the 16S and metagenomic shotgun sequencing datasets that our lab has generated by providing a source of whole genomes for known and novel species of bacteria. Towards the goal of whole genome sequencing the IUMP isolates and submitting any novel strains to metagenomic reference databases, we have grown a total of 260 isolates whose percent sequence similarity to a type strain fall below the traditional threshold for species identification: 98% sequence similarity. [43] 22 of those strains bore no similarity to any type strain present in the reference database and may represent entirely novel taxa. Whole genome sequencing of a few isolates was performed at the IU School of Medicine's Genomics Core, but the genome sequences have not yet been annotated. More rigorous phylogenetic analysis of the sequenced 16S alleles by the Dong Group at Loyola University Chicago is underway. Once complete, those analyses will guide selection of isolates to push forward for whole genome sequence.

In addition to potentially novel species of bacteria, the collection contains high-value clinical representatives of bacteria that are active targets of research like *Prevotella* spp., *Corynebacterium* spp., *Fusobacterium* sp., *Haemophilus* spp., and *Gardnerella* spp. By establishing that the male urethra can support colonization with *Gardnerella* bacteria belonging to one or more of the BV-associated clades with cultured isolates, I have demonstrated that men could potentially transmit live strains during penile-vaginal sex to a female partner. *Prevotella bivia* is also under investigation for its role in BV pathogenesis. [1, 3, 29] Certain species of *Corynebacterium* and *Haemophilus* species

have been observed multiple times in cases of male urethritis. [27, 28, 59] The isolate collection contains multiple representatives of both genera that do not belong to any species based on % sequence similarity to the nearest type strain and may be used to determine how strains associated with urethritis in men compare to strains colonizing other body sites. I grew only one *Fusobacterium* strain, but members of the genus are often pathogenic, and they are commonly isolated from the oral cavity. Hopefully, these isolates will continue to support ongoing research in our laboratory and in others.

The high relative frequency of *Streptococcus* and *Staphylococcus* species is not surprising. Both genera are two of the most abundant members of the human skin flora. Many of the species identified are resident commensals in various regions of the human microbiome. [87] Though it is tempting to assign significance to the fact that strains found in the oral cavity were also cultured from patient samples of men with IU, there are many reasons why bacteria would be present in both locations, especially since the distal urethra and oral cavity are both mucosal surfaces. There is evidence from previous studies, however, supporting an association between unprotected oral sex and male urethritis, particularly among MSM. [6, 64]

There was no one species of bacteria common to all of the IU patients. Preliminary data from the phylogenetic analysis does not point to any particular cluster of novel strains responsible for causing IU in our participants. It may be that rather than one or two mysterious novel species causing a large percentage of urethritis cases, there are several species causing sporadic cases of urethritis.

The present study was weakened by the absence of a companion strain collection from a control cohort of men without urethritis. An open question in IU research is

whether the syndrome is caused by one or more unrecognized pathogens or if the syndrome is the result of a general shift in the bacterial composition of the urethra. The 16S and metagenomic shotgun datasets can offer insight into this question but are unsupported by cultured isolates.

Another weakness is apparent in the large number of strains that did not amplify with either of the 16S rRNA primer sets. It is possible that by only utilizing a single broad-range primer set we missed taxa that would contribute further to our understanding of the urethral microbiome. There were several taxa present in the metagenomic datasets that did not appear in the strain collection. Additionally, bacterial culture cannot detect pathogens from other domains. I am aware of at least two viral pathogens associated with urethritis, and *T. vaginalis* is protozoan. Fortunately, shotgun sequencing is capable of cross-domain coverage. It is also possible that we excluded a pool of participants by requiring five or more PMNLs, which is higher that the CDC's recommended diagnostic criterion of two or more PMNLs. Alternative methods of 16S PCR may have served to increase the number of successfully amplicons. If a round of PCR failed, we immediately went back into the patient sample for new template DNA. Another option would have been to recycle and re-amplify the PCR product.

IU patients enrolled in the study are given the standard treatment of a 1 g dose of azithromycin at the end of their baseline visit. [85] 80% of the IU patients experienced clearance of symptoms and inflammation by the follow-up visit one month later (data not shown). The efficacy of the macrolide antibiotic in clearing IU is evidence of a bacterial etiology.

Though the initial phase of this culture project has concluded, whole genome sequencing efforts are ongoing. If they are fruitful, we will have cultured and sequenced the genomes of dozens of novel bacterial species. My goal is to contribute to a better understanding of IU in the hopes that improved information will guide clinicians to more appropriate and effective patient education and treatment for this highly prevalent syndrome.

References

1. Ahmed, A., Earl, J. Retchless, A., Hillier, S. L., Rabe, L. K., Cherpes, T. L., Powell, E., Janto, B., Eutsey, R., Hiller, N. L., Boissy, R., Dahlgren, M. E., Hall, B. G., Costerton, J. W., Post, J. C., Hu, F. Z., & Ehrlich, G. D. (2012). Comparative genomic analyses of 17 clinical isolates of *Gardnerella vaginalis* provide evidence of multiple genetically isolated clades consistent with subspeciation into genovars. *Journal of bacteriology,194*(15), 3922-3937. doi: 10.1128/JB.00056-12
2. Bachmann, L. H., Manhart, L. E., Martin, D. H., Seña, A. C., Dimitrakoff, J., Skov-Jensen, J., & Gaydos, C. A. (2015). Advances in the understanding and treatment of male urethritis. *Clinical infectious diseases, 61*(S8), S763-S769. https://doi.org/10.1093/cid/civ755
3. Balashov, S. V., Mordechai, E., Adelson, M. E., & Gygax, S. E. (2014). Identification, quantification, and subtyping of *Gardnerella vaginalis* in noncultured clinical vaginal samples by quantitative PCR. *Journal of medical microbiology, 63*(2), 162-175. doi: 10.1099/jmm.0.066407-0
4. Bowie, W. R., Pollock, H. M., Forsyth, P. S., Floyd, J. F., Alexander, E. R., Wang, S. P., & Holmes, K. K. (1977). Bacteriology of the urethra in normal men and men with nongonococcal urethritis. *Journal of clinical microbiology*, *6*(5), 482–488.
5. Bowie, W. R., Wang, S. P., Alexander, E. R., Floyd, J., Forsyth, P. S., Pollock, H. M., Lin, J. S., Buchanan, T. M., & Holmes, K. K. (1977). Etiology of nongonococcal urethritis: evidence for Chlamydia trachomatis and Ureaplasma urealyticum. *The Journal of clinical investigation*, *59*(5), 735–742. https://doi.org/10.1172/JCI108694

6. Bradshaw, C. S., Tabrizi, S. N., Read, T. R. H., Garland, S. M., Hopkins, C. A., Moss, L. M., & Fairley, C. K. (2006). Etiologies of nongonococcal urethritis: bacteria, viruses, and the association with orogenital exposure. *The journal of infectious diseases*, *193*(3), 336–345. https://doi.org/10.1086/499434

7. Braxton, J., Davis, D., Flagg, E., Grey, J., Grier, L., Harvey, A., Kidd, S., Kreisel, K., Llata, E., Presley, R., Ramirez, V., Shapiro, S., Stenger, M., Torrone, E., Weinstock, H., & Weston, E. (2017). Sexually transmitted disease surveillance 2016. *Centers for Disease Control and Prevention. U.S. Department of Health and Human Services.*

8. Buttigieg, G. (1985). Cervicitis and urethritis caused by group B *streptococcus*: case report. *Genitourinary medicine*, *61*(5), 343–344. https://doi.org/10.1136/sti.61.5.343

9. Chambers, L. C., Hughes, J. P., Glick, S. N., Morgan, J. L., Lowens, M. S., Robinson, T. S., Romano, S. S., Leipertz, G. L., Jensen, J. S., Khosropour, C. M., Fredricks, D. N., Golden, M. R., & Manhart, L. E. (2019). Resolution of symptoms and resumption of sex after diagnosis of nongonococcal urethritis among men who have sex with men. *Sexually transmitted diseases 46*(10), 676-682. doi: 10.1097/OLQ.0000000000001040

10. Chanakira, E., O'Cathain, A., Goyder, E. C., & Freeman, J. V. (2014) Factors perceived to influence risky sexual behaviours among university students in the United Kingdom: a qualitative telephone interview study. *BMC public health, 14,* 1055. https://doi.org/10.1186/1471-2458-14-1055

11. CDC Fact Sheet. People who have STDs are more likely to get HIV, when compared to people who do not have STDs. October 2019. https://www.cdc.gov/std/hiv/stdfact-std-hiv-detailed.htm

12. Centers for Disease Control and Prevention. *Sexually Transmitted Disease Surveillance 2018.* U.S. Department of Health and Human Services 2019. doi: 10.15620/cdc.79370

13. Chowdhury, M. N., & Pareek, S. S. (1984). Urethritis caused by group B streptococci: a case report. *The British journal of venereal diseases*, *60*(1), 56–57. https://doi.org/10.1136/sti.60.1.56

14. Cornejo, O. E., Hickey, R. J., Suzuki, H., & Forney, L. J. (2017). Focusing the diversity of *Gardnerella vaginalis* through the lens of ecotypes. *Evolutionary applications*, *11*(3), 312–324. https://doi.org/10.1111/eva.12555

15. Deguchi, T., Yoshida, T., Miyazawa, T., Yasuda, M., Tamaki, M., Ishiko, H., & Maeda, S. (2004). Association of *Ureaplasma urealyticum* (biovar 2) with nongonococcal urethritis. *Sexually transmitted diseases, 31*(3), 192-195. doi: 10.1097/01.OLQ.0000114653.26951.71

16. Deal, C. Cates, W., Peeling, R., & Wald, A. (2004). Long-term clinical sequelae of sexually transmitted infections in women. *Emerging infectious diseases, 10*(11), e2. https://doi.org/10.3201/eid1011.040622_02

17. Deza, G., Martin-Ezquerra, G., Gómez, J., Villar-García, J., Supervia, A., & Pujol, R. M. (2016). Isolation of *Haemophilus influenzae* and *Haemophilus parainfluenzae* in urethral exudates from men with acute urethritis: a descriptive

study of 52 cases. *Sexually transmitted infections*, *92*,29-31. https://doi.org/10.1136/sextrans-2015-052135

18. Dong, Q., Nelson, D. E., Toh, E., Diao, L., Gao, X., Fortenberry, J. D., & Van der Pol, B. (2011). The microbial communities in male first catch urine are highly similar to those in paired urethral swab specimens. *PloS one*, *6*(5), e19709. https://doi.org/10.1371/journal.pone.0019709
19. Dunlop, E. M., Jones, B. R., Darougar, S., & Treharne, J. D. (1972). Chlamydia and non-specific urethritis. *British medical journal*, *2*(5813), 575–577. https://doi.org/10.1136/bmj.2.5813.575
20. Dwari, B. C., Bhatt, M., Mishra, M., Tripathy, N., & Sathpathy, P. K. (2018). Male urethritis with or without discharge: a clinic aetiological study in Hi-tech Medical College and Hospital Bhubaneswar, Odisha. *International journal of research in dermatology*, *4*(4), 484-488. http://dx.doi.org/10.18203/issn.2455-4529.IntJResDermatol20183377
21. Fontaine, E. A., Taylor-Robinson, D., Hanna, N. F., & Coufalik, E. D. (1982). Anaerobes in men with urethritis. *The British journal of venereal diseases*, *58*(5), 321–326. https://doi.org/10.1136/sti.58.5.321
22. Frølund, M., Falk, L., Ahrens, P., & Jensen, J. S. (2019). Detection of *ureaplasma*s and bacterial vaginosis associated bacteria and their association with non-gonococcal urethritis in men. *PloS one*, *14*(4), e0214425. https://doi.org/10.1371/journal.pone.0214425
23. Frølund, M., Lidbrink, P., Wikström, A., Cowan, S., Ahrens, P., & Skov-Jensen, J. (2016). Urethritis-associated pathogens in urine from men non-gonococcal

urethritis: a case-control study. *Acta dermato-venereologica, 96,* 689-694. doi: 10.2340/00015555-2314

24. Frølund, M., Wikström, A., Lidbrink, P., Abu Al-Soud, W., Larsen, N., Harder, C. B., Sørensen, S. J., Jensen, J. S., & Ahrens, P. (2018). The bacterial microbiota in first-void urine from men with and without idiopathic urethritis. *PloS one*, *13*(7), e0201380. https://doi.org/10.1371/journal.pone.0201380

25. Fredricks, D. N., Fiedler, & T. L., Marrazzo, J. M. (2005). Molecular identification of bacteria associated with bacterial vaginosis. *The New England journal of medicine, 353*(18), 1899-1911. doi: 10.1056/NEJMoa043802

26. Fredricks, D. N., Fiedler, T. L., Thomas, K. K., Oakley, B. B., & Marrazzo, J. M. (2007). Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. *Journal of clinical microbiology*, *45*(10), 3270–3276. https://doi.org/10.1128/JCM.01272-07

27. Galan-Sanchez, F., Aznar-Marin, P., Marin-Casanova, P., Garcia-Martos, P., & Rodriguez-Iglesias, M. (2011). Urethritis due to *Corynebacterium glucuronolyticum. Journal of infection and chemotherapy, 17,* 720-721. https://doi.org/10.1007/s10156-011-0237-y

28. Gherardi, G., Di Bonaventura, G., Pompilio, A., & Savini, V. (2015). *Corynebacterium glucuronolyticum* causing genitourinary tract infection: Case report and review of the literature. *IDCases, 2*(2), 56–58. https://doi.org/10.1016/j.idcr.2015.03.001

29. Gilbert, N. M., Lewis, W. G., Li, G., Sojka, D. K., Lubin, J. B., & Lewis, A. L. (2019). *Gardnerella vaginalis* and *Prevotella bivia* trigger distinct and overlapping

phenotypes in a mouse model of bacterial vaginosis. *The journal of infectious diseases 220*(7), 1099-1108. https://doi.org/10.1093/infdis/jiy704

30. Hill, J. E., Albert, A. Y. K., & VOGUE Research Group. (2019). Resolution and co-occurrence patters of *Gardnerella leopoldii, G. swidsinkii, G. piotii,* and *G. vaginalis* within the vaginal microbiome. *Infection and immunity, 87*(12), e00532-19. doi: 10.1128/IAI.00532-19
31. Holmes, K. K., Handsfield, H. H., Wang, S. P., Wentworth B. B., Turck, M., Anderson, J. B., & Alexander E. R. (1975). Etiology of nongonococcal urethritis. *New England journal of medicine 292*(23), 1199-1205. doi: 10.1056/NEJM197506052922301
32. Holmes, K. K., Levine, R., & Weaver, M. (2004). Effectiveness of condoms in preventing sexually transmitted infections. *Bulletin of the World Health Organization, 82*(6), 454-461.
33. Hook, E. W., 3rd, & Kirkcaldy, R. D. (2018). A Brief History of Evolving Diagnostics and Therapy for Gonorrhea: Lessons Learned. *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America, 67*(8), 1294–1299. https://doi.org/10.1093/cid/ciy271
34. Horie, K., Ito, S., Hatazaki, K., Yasuda, M., Nakano, M., Kawakami, K., Fujita, Y., Ito, M., Ezaki, T., & Deguchi, T. (2017). *Haemophilus quentini* in the urethra of men complaining of urethritis symptoms. *Journal of infection and chemotherapy 24*(1), 71-74. http://dx.doi.org/10.1016/j.jiac.2017.08.007

35. Horner, P. (2011) The etiology of acute nongonococcal urethritis – the enigma of idiopathic urethritis? *Sexually transmitted diseases, 38*(3), 187-189. doi: 10.1097/OLQ.0b013e318207c2eb

36. Horner, P. J. & Martin, D. H. (2017) *Mycoplasma genitalium* infection in men. *The Journal of infectious diseases, 216*(suppl_2), S396-S405. https://doi.org/10.1093/infdis/jix145

37. Hsu, M. S., Wu, M. Y., Lin, T. H., & Liao, C. H. (2012). *Haemophilus parainfluenzae* urethritis among homosexual men. *Journal of microbiology, immunology, and infection 48*(4), 450-452. http://dx.doi.org/10.1016/j.jmii.2012.11.007

38. Ingianni, A., Petruzzelli, S., Morandotti, G., Pompei, R. (1997). Genotypic differentiation of *Gardnerella vaginalis* by amplified ribosomal DNA restriction analysis (ARDRA). *FEMS Immunology & Medical Microbiology 18*(1), 61–66. https://doi.org/10.1111/j.1574-695X.1997.tb01028.x

39. Ito, S., Hanaoka, N., Shimuta, K., Seike, K., Tsuchiya, T., Yasuda, M., Yokoi, S., Nakano, M., Ohnishi, M., & Deguchi, T. (2016). Male non-gonococcal urethritis: from microbiological etiologies to demographic and clinical features. *International journal of urology 23,* 325-326. doi: 10.1111/iju.13044

40. Ito, S., Hatazaki, K., Shimuta, K., Kondo, H., Mizutani, K., Yasuda, M., Nakane, K., Tsuchiya, T., Yokoi, S., Nakano, M., Ohinishi, M., & Deguchi, T. (2017). *Haemophilus influenzae* isolated from men with acute urethritis. *Sexually transmitted diseases 44*(4), 205-210. https://doi.org/10.1097/OLQ.0000000000000573

41. Janulaitiene, M., Gegzna, V., Baranauskiene, L., Bulavaitė, A., Simanavicius, M., & Pleckaityte, M. (2018). Phenotypic characterization of Gardnerella vaginalis subgroups suggests differences in their virulence potential. *PloS one*, *13*(7), e0200625. https://doi.org/10.1371/journal.pone.0200625

42. Janulaitiene, M., Paliulyte, V., Grinceviciene, S., Zakareviciene, J., Vladisauskiene, A., Marcinkute, A., & Pleckaityte, M. (2017). Prevalence and distribution of Gardnerella vaginalis subgroups in women with and without bacterial vaginosis. *BMC infectious diseases*, *17*(1), 394. https://doi.org/10.1186/s12879-017-2501-y

43. Johnson, J.S., Spakowicz, D.J., Hong, B. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nature Communications 10*, 5029. https://doi.org/10.1038/s41467-019-13036-1

44. Jordan, S. J., Aaron, K. J., Schwebke, J. R., Van Der Pol, B. J., & Hook, E. W., 3rd (2018). Defining the urethritis syndrome in men using patient reported symptoms. *Sexually transmitted diseases*, *45*(7), e40–e42. https://doi.org/10.1097/OLQ.0000000000000790

45. Jordan S. J., Toh E., Williams J. A., Fortenberry, L., LaPradd, M., Katz, B. P., Batteiger, B. E., Nelson, D. E., & Batteiger, T. A. (2019). Aetiology and prevalence of mixed-infections and mono-infections in non-gonococcal urethritis in men: a case-control study. *Sexually transmitted Infections*. doi: 10.1136/sextrans-2019-054121

46. Koroglu, M., Yakupogullari, Y., & Aydogan, F. (2007). A case of urethritis due to *Streptococcus pneumoniae*. *Sexually transmitted diseases, 34*(12), 1040. doi: 10.1097/OLQ.0b013e31815b0168

47. Krieger, J. N., Riley, D. E., Roberts, M. C., & Berger, R. E. (1996). Prokaryotic DNA sequences in patients with chronic idiopathic prostatitis. *Journal of clinical microbiology*, *34*(12), 3120–3128.

48. Lafferty, W. E., Hughes, J. P., & Handsfield H. H. (1997). Sexually transmitted diseases in men who have sex with men: acquisition of gonorrhea and nongonococcal urethritis by fellatio and implications for STD/HIV prevention. *Sexually transmitted diseases, 24*(5), 272-278.

49. Lane, D. J. (1991) 16S/23S rRNA sequencing. In E. Stackebrandt & M. Goodfellow (Eds.), *Nucleic acids techniques in bacterial systematics* (pp. 115–147). John Wiley & Sons.

50. Liu, C. M., Hungate, B. A., Tobian, A. A., Ravel, J., Prodger, J. L., Serwadda, D., Kigozi, G., Galiwango, R. M., Nalugoda, F., Keim, P., Wawer, M. J., Price, L. B., & Gray, R. H. (2015). Penile Microbiota and Female Partner Bacterial Vaginosis in Rakai, Uganda. *mBio, 6*(3), e00589. https://doi.org/10.1128/mBio.00589-15

51. Maeda, S. I., Yasuda, M., Ito, S., Seike, K., Ito, S. I., & Deguchi, T. (2009). Azithromycin treatment for nongonococcal urethritis negative for *Chlamydia trachomatis, Mycoplasma genitalium, Mycoplasma hominis, Ureaplasma parvum*, and *Ureaplasma urealyticum. International journal of urology, 16*(2), 215-216. https://doi.org/10.1111/j.1442-2042.2008.02196.x

52. Manhart, L. E., Khosropour, C. M., Liu, C., Gillespie, C. W., Depner, K., Fiedler, T., Marrazzo, J. M., & Fredricks, D. N. (2013). Bacterial vaginosis-associated bacteria in men: association of *Leptotrichia/Sneathia* spp. with nongonococcal urethritis. *Sexually transmitted diseases*, *40*(12), 944–949. https://doi.org/10.1097/OLQ.0000000000000054

53. Mazuecos, J., Aznar, J., Rodriguez-Pichardo, A., Marmesat, F., Borobio, M., Perea, E. & Camacho, F. (1998), Anaerobic bacteria in men with urethritis. *Journal of the European academy of dermatology and venereology*, *10*, 237-242. doi:10.1111/j.1468-3083.1998.tb00743.x

54. Montagnini-Spaine, D., Mamizuka, E. M., Pereira-Cedenho, A., & Srougi, M. (2000). Microbiologic aerobic studies on normal male urethra. *Urology 56*(2), 207-210.

55. Nayyar, K. C., O'Neill, J. J., Hambling M. H., & Waugh, M. A. (1976). Isolation of Chlamydia trachomatis from women attending a clinic for sexually transmitted diseases. *British journal of venereal diseases 52,* 396-398.

56. Nelson, D. E., Van Der Pol, B., Dong, Q., Revanna, K. V., Fan, B., Easwaran, S., Sodergren, E., Weinstock, G. M., Diao, L., & Fortenberry, J. D. (2010). Characteristic male urine microbiomes associate with asymptomatic sexually transmitted infection. *PloS one*, *5*(11), e14116. https://doi.org/10.1371/journal.pone.0014116

57. Noble R. C. (1985). Colonisation of the urethra with *Streptococcus pneumoniae*: a case report. *Genitourinary medicine*, *61*(5), 345–346. https://doi.org/10.1136/sti.61.5.345

58. Piot, P., Van Dyck, E., Peeters, M., Hale, J., Totten, P. A., & Holmes, K. K. (1984). Biotypes of Gardnerella vaginalis. *Journal of clinical microbiology, 20*(4), 677–679.

59. Quentin, R., Ruimy, R., Rosenau, A., Musser, J. M., & Christen, R. (1996). Genetic identification of cryptic genospecies of Haemophilus causing urogenital and neonatal infections by PCR using specific primers targeting genes coding for 16S rRNA. *Journal of clinical microbiology, 34*(6), 1380–1385.

60. Rane, V. S., Fairley, C. K., Weerakoon, A., Read, T. H., Fehler, G., Chen, M. Y., & Bradshaw, C. S. (2014). Characteristics of acute nongonococcal urethritis in men differ by sexual preference. *Journal of clinical microbiology, 52*(8), 2971–2976. https://doi.org/10.1128/JCM.00899-14

61. Riemersma, W. A., van der Schee, C. J., van der Meijden, W. I., Verbrugh, H. A., & van Belkum, A. (2003). Microbial population diversity in the urethras of healthy males and males suffering from nonchlamydial, nongonococcal urethritis. *Journal of clinical microbiology, 41*(5), 1977–1986. https://doi.org/10.1128/jcm.41.5.1977-1986.2003

62. Takahashi, S., Matsukawa, M., Kurimura, Y., Takeyama, K., Kunishima, Y., Iwasawa, A., Koroku, M., Tanda, H., Suzuki, N., Takagi, Y., Hirose, T., Nishimura, M., & Tsukamoto, T. (2008). Clinical efficacy of azithromycin for male nongonococcal urethritis. *Journal of infection and chemotherapy, 14,* 409-412. doi: 10.1007/s10156-008-0643-y

63. Taylor-Robinson, D., & Horner, P. J. (2001). The role of *Mycoplasma genitalium* in in non-gonococcal urethritis. *Sexually transmitted infections, 77,* 229-231. doi: 10.1136/sti.77.4.229

64. Saxena, A., Dawar, R., & Bora, U. (2018). *Haemophilus parainfluenzae* urethritis through orogenital transmission. *Indian journal of sexually transmitted diseases and AIDS, 39*(2), 127–129. https://doi.org/10.4103/ijstd.IJSTD_45_17

65. Schlay, J., McClung, M., Patnaik, J. L., Douglas, & J. M. (2004). Comparison of sexually transmitted disease prevalence by reported condom use: errors among consistent condom users seen at an urban sexually transmitted disease clinic. *Sexually transmitted diseases, 31*(9), 526-532. doi: 10.1097/01.olq.0000137897.17919.d1

66. Schwebke, J. R., Rivers, C., & Lee, J. (2009). Prevalence of *Gardnerella vaginalis* in male sexual partners of women with and without bacterial vaginosis. *Sexually transmitted diseases, 36*(2), 92-94. doi: 10.1097/OLQ.0b013e3181886727

67. Schwebke, J. R., Rompalo, A., Taylor, S., Seña, A. C., Martin, D. H., Lopez, L. M., Lensing, S., & Lee, J. Y. (2011). Re-evaluating the treatment of nongonococcal urethritis: emphasizing emerging pathogens--a randomized clinical trial. *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America, 52*(2), 163–170. https://doi.org/10.1093/cid/ciq074

68. Schwartz, M. A., Lafferty, W. E., Hughes, J. P., & Handsfield, H. H. (1997). Risk factors for urethritis in heterosexual men: the role of fellatio and other sexual practices. *Sexually transmitted diseases, 24*(8), 449-455.

69. Serour, F., Samra, Z., Kushel, Z., Gorenstein, A., & Dan, M. (1997). Comparative periurethral bacteriology of uncircumcised and circumcised males. *Genitourinary medicine*, *73*(4), 288–290. https://doi.org/10.1136/sti.73.4.288

70. Shelburne, S. A., Sahasrabhojane, P., Saldana, M., Yao, H., Su, X., Horstmann, N., Thompson, E., & Flores, A. R. (2014). Streptococcus mitis strains causing severe clinical disease in cancer patients. *Emerging infectious diseases*, *20*(5), 762–771. https://doi.org/10.3201/eid2005.130953

71. Shipitsyna, E., Krysanova, A., Khayrullina, G., Shalepo, K., Savicheva, A., Guschin, A., & Unemo, M. (2019). Quantitation of all Four Gardnerella vaginalis Clades Detects Abnormal Vaginal Microbiota Characteristic of Bacterial Vaginosis More Accurately than Putative G. vaginalis Sialidase A Gene Count. *Molecular diagnosis & therapy*, *23*(1), 139–147. https://doi.org/10.1007/s40291-019-00382-5

72. Srinivasan, S., Hoffman, N. G., Morgan, M. T., Matsen, F. A., Fiedler, T. L., Hall, R. W., Ross, F. J., McCoy, C. O., Bumgarner, R., Marrazzo, J. M., & Fredricks, D. N. (2012). Bacterial communities in women with bacterial vaginosis: high resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. *PloS one*, *7*(6), e37818. https://doi.org/10.1371/journal.pone.0037818

73. Srinivasan, S., Munch, M. M., Sizova, M. V., Fiedler, T. L., Kohler, C. M., Hoffman, N. G., Liu, C., Agnew, K. J., Marrazzo, J. M., Epstein, S. S., & Fredricks, D. N. (2016). More easily cultivated than identified: classical isolation with molecular identification of vaginal bacteria. *The Journal of infectious diseases*, *214 Suppl 1*(Suppl 1), S21–S28. https://doi.org/10.1093/infdis/jiw192

74. Stover, J., Rosen, J. E., Carvalho, M. N., Korenromp, E. L., Friedman, H. S., Cogan, M., & Deperthes, B. (2017). The case for investing in the male condom. *PloS one*, *12*(5), e0177108. https://doi.org/10.1371/journal.pone.0177108

75. Sturm, A. W. (1986). *Haemophilus infleunzae* and *Haemophilus parainfluenzae* in nongonococcal urethritis. *The journal of infectious diseases, 153*(1), 165-167. https://doi.org/10.1093/infdis/153.1.165

76. Takahashi, S., Matsukawa, M., Kurimura, Y., Takeyama, K., Kunishima, Y., Iwasawa, A., Koroku, M., Tanda, H., Suzuki, N., Takagi, Y., Hirose, T., Nishimura, M., & Tsukamoto, T. (2008). *Journal of infection and chemotherapy*, *14*, 409-412. https://doi.org/10.1007/s10156-008-0643-y

77. Tabrizi, S. N., Ling, A. E., Bradshaw, C. S., Fairley, C. K., & Garland, S. M. (2007). Human adenoviruses types associated with non-gonococcal urethritis. *Sexual health 4*, 41-44. https://doi.org/10.1071/SH06054

78. Territo, H., & Ashurst, J., V. (2020). Nongonococcal urethritis (NGU). [Updated 2019 December 17]. In: StatPearls. Available from: https://www.ncbi.nlm.nih.gov/books/NBK535411//

79. Truong, D., Franzosa, E., Tickle, T. L., Scholz, M., Weingart, G., Pasolli, E., Tett, A., Huttenhower, C., & Segata, N. (2015). MetaPhlAn2 for enhanced metagenomic taxonomic profiling. *Nature methods, 12*, 902–903. https://doi.org/10.1038/nmeth.3589

80. Vaneechoutte, M., Guschin, A., Van Simaey, L., Gansemans, Y., Van Nieuwerburgh, F., & Cools, P. (2019). Emended description of *Gardnerella vaginalis* and a description of *Gardnerella leopoldii* sp. nov., *Gardnerella piotii* sp.

nov. and *Gardnerella swidinskii* sp. nov., with delineation of 13 genomic species within the genus *Gardnerella. International journal of systemic and evolutionary microbiology.* doi: 10.1099/ijsem.0.003200

81. Warner, L., Newman, D. R., Austin, H. D., Kamb, M. L., Douglas, J. M., Malotte, C. K., Zenilman, J. M., Rogers, J., Bolan, G., Fishbein, M., Kleinbaum, D. G., Macaluso, M., & Peterman, T. A. (2004). Condom effectiveness for reducing transmission of Gonorrhea and Chlamydia: the importance of assessing partner infection status. *American journal of epidemiology*, *159*(3), 242-251. https://doi.org/10.1093/aje/kwh044

82. Wetmore, C. M., Manhart, L. E., & Golden, M. R. (2009). Idiopathic urethritis in young men in the United States: prevalence and comparison to infections with known sexually transmitted pathogens. *The Journal of adolescent health: official publication of the Society for Adolescent Medicine*, *45*(5), 463–472. https://doi.org/10.1016/j.jadohealth.2009.06.024

83. Wetmore, C. M., Manhart, L. E., Lowens, M. S., Golden, M. R., Jensen, N. L., Astete, S. G., Whittington, W. L., & Totten, P. A. (2011). Ureaplasma urealyticum is associated with nongonococcal urethritis among men with fewer lifetime sexual partners: a case-control study. *The Journal of infectious diseases*, *204*(8), 1274–1282. https://doi.org/10.1093/infdis/jir517

84. Wetmore, C. M., Manhart, L. E., Lowens, M. S., Golden, M. R., Whittington, W. L., Xet-Mull, A. M., Astete, S. G., McFarland, N. L., McDougal, S. J., & Totten, P. A. (2011). Demographic, behavioral, and clinical characteristics of men with nongonococcal urethritis differ by etiology: a case-comparison study. *Sexually*

transmitted diseases, *38*(3), 180–186. https://doi.org/10.1097/OLQ.0b013e3182040de9

85. Willeén, M., Hoist, E., Myhre, E. B., & Olsson, A. M. (1996). Bacterial flora of the genitourinary tract in healthy fertile men. *Scandinavian journal of urology and nephrology 30*(5), 387-393. doi:10.3109/00365599609181315

86. Worworksi, K. A., Bolan, G. A. (2015). Sexually transmitted diseases treatment guidelines, 2015. *CDC Morbidity and mortality weekly report: recommendations and reports 64*(3). https://www.cdc.gov/std/tg2015/tg-2015-print.pdf

87. Yoon, S. H., Ha, S. M., Kwon, S., Lim, J., Kim, Y., Seo, H., & chun, J. (2017) Introducing EZBioCloud: a taxonomically united database of 16S rRNA and whole genome assembles. *Int J Syst Evol Microbiol*, *67*, 1613-1617. https://doi.org/10.1099/ijsem.0.001755

88. Zozaya, M., Ferris, M. J., Siren, J. D., Lillis, R., Myers, L., Nsuami, M. J., Eren, A. M., Brown, J., Taylor, C. M., & Martin, D. H. (2016). Bacterial communities in penile skin, male urethra, and vaginas of heterosexual couples with and without bacterial vaginosis. *Microbiome*, *4*, 16. https://doi.org/10.1186/s40168-016-0161-6

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